Spore-forming bacteria as a proxy for the reconstruction of past environment and possible use for the detection of antibiotic resistance genes in the environment

Thèse présentée à la Faculté des Sciences
Université de Neuchâtel
Institut de Biologie

Pour l'obtention du grade de
DOCTEUR ÈS SCIENCES

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Soutenue le 18 novembre 2019

Université de Neuchâtel
2019

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Neuchâtel, le 16 décembre 2019 Le Doyen, Prof. P. Felber

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Acknowledgements

- Pilar Junier, my supervisor, for her confidence, help, support, enthusiasm, and for putting up with me and my obsessional personality during these past four years. Thanks for giving me the opportunity to realize this work and to believe in me.
- Anaël Lehmann, for the very nice collaboration, easy taking and always in a good mood.
- Torsten Vennemann, Daniel Ariztegui and Eric Verrecchia, for their expertise and time.
- Jean-Luc Loizeau, for his contribution to the dating analyses.
- Helmut Bürgmann and Karin Beck from the Eawag, for their contribution and expertise.
- Casey Hubert, Daniel Croll and Jakob Zopfi for accepting to be part of my committee thesis, for their wise comments and advices.
- Kurt Hanselmann, for his great help on the field trip in Graubünden, and for sharing his knowledges of the Joeri lakes.
- Matthieu Bueche and Vincent Hervé, for their help and advice, and sharing their codes and data.
- Zhanna Bayrychenko and Laura Madueño, for their great work.
- Mathilda Fatton, for all the work accomplished, her contribution to the field and laboratory work.
- Isha Hashmi, Wafa Kooli, Frédéric Sandoz and Maxime Fatton for their great help on the field, putting their lives on the line for a few sediment samples.
- Ilona Palmieri and Andrea Corona Ramirez for the full of DNA extraction performed in record time.
- Nicole Jeanneret, for her kindness, time, help and advices.
- All the peoples from the lab, for their kindness and good mood.
- And of course my family, Vanessa and Romain, who brightened my days. My parents Monique and Jean-Noël, for their continued support over the years.
- All the peoples I may have forgotten here, who helped me in any possible way to achieve this work and that have done this past four years fantastic.

Contents

Summaries

Summary

Paleoecology is the study of past environments based on the analysis of sedimentary records and their chemical, isotopic and biological composition. Paleoecology aims to establish the relationship between organisms and their physical and chemical environments, in order to assess the causes and rate of ecological change and to reconstruct ecosystem history. Such studies allow for a better understanding of climate variability, range of natural fluctuations, extreme events frequency and/or anthropogenic impact over time, at various time scales. Paleoecological indicators include a wide variety of compounds and structures including mineralogical, chemical and isotopic composition of sediment particles, as well as biological structures such as pollen grain or microfossils (diatoms, ostracods). Environmental DNA has also been proposed as a biological proxy, but DNA-based methods are dependent on DNA conservation. To be applicable, such proxies must be preserved in sediments for long-time periods. That must be the case for bacterial endospores or other spore-like structures. These highly specialized cellular forms allow the organisms able to produce them to resist and survive harsh environmental conditions by entering a dormant state. Such structures have long been proposed as paleoecological proxies, but only the recent development of molecular methods allowing their study results in their potential widespread application for paleoecology. The new-developed methods include notably a DNA extraction protocol adapted to highly resistant structures, and a treatment for the enrichment of spores (spore-separation method). The nature of the spore-separation treatment, which consists in the enrichment of cells able to withstand a harsh lysis method, does not provide direct evidence for the formation of specialized cells, we will use the term “lysis-resistant” instead of spores when appropriate.

The first part of this thesis aimed to evaluate the potential of using bacterial DNA, from both the total and the lysis-resistant community, as a proxy for the reconstruction of past environments. First, the efficiency of the newly developed method for the isolation of spores was assessed by comparing the total and the lysis-resistant community. The community composition was compared in samples that have or not been submitted to the spore-separation treatment. The results showed that the lysis-resistant community possess a unique signature compared to the total community. Interestingly, various genera hitherto considered as non-sporulating were found in high abundance in the lysis-resistant community but were not among the most abundant genera in the total community. This demonstrates a capacity to resist the spore-separation treatment and strongly suggests the ability in these groups of the production of a lysis-resistant structure. Second, the separation method was used to investigate the diversity, distribution, and community structure of potential spore-forming organisms in the environment. To that purpose, data was obtained from four contrasting study sites. The results of our analysis revealed an unsuspected diversity of organisms in the lysis-resistant community. The lysis-resistant community also showed a geographic distribution pattern, challenging a hypothetical cosmopolitan distribution of spore-formers. Results of this study suggest that the ability to form spores or similar resting and durable cell structures is more widespread than previously suspected.

In a second study, the application of these novel methods was tested for the reconstruction of the history of the ephemeral Lake Liambezi (Namibia). Using a multidisciplinary approach including the use of bacterial DNA in complement to geochemical and sedimentological analyses, the climate evolution over the past 5500 years was reconstructed. This highlighted an alternation of dry and wet periods,

and changes in the hydrological lake regime (from fen to lake). DNA was isolated from both the total and the lysis-resistant fraction of the community, both of which reflected changes in the environmental conditions, demonstrating their relevancy for paleoecological studies. Interestingly, in some cases, putative environmental conditions, biological processes, or extreme events were only seen in one fraction of the community, highlighting the complementarity of investigating both fractions of the community. In addition, the analysis of the bacterial community and specific populations helped to elaborate a coherent age model relating the three sediment cores studied. This analysis suggested hydrothermal activity and sulfur cycling within the lake. Results of this study demonstrated the potential of using bacterial DNA (total and/or spores) to identify changes and variability in the environmental conditions, and the strengths of a multidisciplinary approach to reconstruct ecosystem history.

The second part of this thesis aimed to evaluate the possible use of these new-developed methods for studying the prevalence of antibiotic resistance genes (ARG) in the environment. Since the discovery of penicillin by Fleming in 1928 and the development of antibiotics for medical purpose from the 1940s', the extensive use of antibiotics has created a selection pressure that has contributed to the emergence of antibiotic resistant bacteria (ARB) and multi-resistant bacteria (MRB). The multiplication of such organisms represents a real threat for human health in the future. Long ignored, this problem is now considered as urgent. Although the distribution and frequency of ARG in the environment is widely unknown, it is clear that human activities have an impact in prevalence. In this part of the thesis, the use of DNA extracted from lysis-resistant bacterial cells for tracking ARG in the environment was assessed.

In a first study, the accumulation of ARG over time was investigated, in both the total and the lysis-resistant community, in sediments from Lake Geneva (CH). The results showed that two selected ARG (*tet*(W) and *sul*1) could be detected in both the lysis-resistant and the total fraction of the community. Both genes were found in higher frequency (copies/ng DNA) in the lysis-resistant community of the community compared to the total community, suggesting the lysis-resistant fraction was enriched in ARG. Accumulation patterns of these two ARG showed to be correlated to the historical use of their related antibiotics. When investigating the relationship between the ARG accumulation and the bacterial community composition, each gene appeared to be correlated to different taxonomic group. While *tet*(W) was mainly correlated to a change in the relative abundance in the Firmicutes, *sul*1 was correlated to a more diverse group of organisms, suggesting both ARG are differently distributed across bacterial taxa. These results show that spore-like structures can be used to trace back the effect of historical usage of antibiotics on resistance prevalence.

In a second study, the impact of wastewater release on the environmental spread of ARG associated to the lysis-resistant community was investigated in wastewater-impacted sediments from the Vidy Bay (CH). The wastewater treatment plant (WWTP) was identified as a source of ARG. The two studied ARG (*tet*(W) and *sul*1) were detected in all samples, and their abundance/frequency decreased with increasing distance to the WWTP outlet. ARG levels were correlated to other indicators of wastewater discharge, such as C_{org}, N_{tot} and DNA. Both ARG showed to be differently enriched in the lysis-resistant community. Similarly to what had been observed in the first study, *tet*(W) frequency mainly correlated with the relative abundance of genera belonging to the Firmicutes (*Clostridium* and *Ruminococcus)*, and *sul1* frequency correlated with a large taxonomic spectrum of organisms. The high relative abundance of *Clostridium* spp., coupled to its correlation with *tet*(W) frequency, suggested that members of this genus might be a potential vector for *tet*(W) dissemination.

These two studies constituted the first evidence of the possible detection of ARG in spores or lysis-resistant structures. DNA extracted from these resilient structures appeared to be a good proxy for assessing the dispersal and the accumulation of ARG over time in environmental samples. Given their high survival ability and propensity for dispersion, this lysis-resistant fraction of the community might receive more attention in the future, for a better understanding of its role in the fate of ARG, and to help implementing appropriate usage management strategies to halt antibiotic resistance and improve their removal during waste treatment.

Keywords: spores, lysis-resistant, paleoecology, antibiotic resistance, bacterial community, sediment.

Résumé

La paléoécologie est l'étude des environnements passés, basée sur l'analyse d'archives sédimentaires et de leur composition chimique, isotopique et biologique. La paléoécologie cherche à établir le lien entre les organismes et leur environnement physique et chimique, afin de déterminer la cause et fréquence des changements écologiques, et de reconstruire l'histoire des écosystèmes. De telles études permettent de mieux évaluer et comprendre la variabilité du climat, l'ampleur des fluctuations naturelles, la fréquence des évènements extrêmes, et/ou l'impact anthropique. Les indicateurs paléoécologiques comprennent une grande variété de composés et de structures, dont la composition minéralogique, chimique et isotopique des sédiments, ainsi que des structures biologiques telles que les grains de pollen ou les microfossiles (diatomées, ostracodes). L'ADN environnemental a également été proposé comme marqueur biologique, mais les méthodes basées sur l'ADN sont dépendantes de sa conservation. Pour être utilisables, de tels marqueurs doivent être conservés dans les sédiments pendant de longues périodes, ce qui devrait être le cas des endospores bactériennes ou d'autres structures semblables à des spores. Ces formes cellulaires hautement spécialisées permettent aux organismes capables de les produire de résister et de survivre à des conditions environnementales extrêmes en entrant dans un état de dormance. De telles structures ont été proposées depuis longtemps comme marqueurs paléoécologiques, mais ce n'est que récemment que des méthodes moléculaires permettant de les étudier ont été développées, ouvrant la voie à une utilisation plus large en paléoécologie. Les nouvelles méthodes développées comprennent notamment un protocole d'extraction d'ADN adapté aux structures très résistantes, et un traitement d'enrichissement des spores (méthode de séparation des spores). La nature du traitement de séparation des spores, qui consiste à enrichir des cellules capables de résister à une méthode de lyse agressive, ne fournit pas de preuve directe de la formation de cellules spécialisées, nous utiliserons donc le terme « résistant(e) à la lyse » au lieu de spores le cas échéant.

L'objectif de la première partie de cette thèse était d'évaluer la possible utilisation de l'ADN bactérien, provenant de la communauté totale et de la communauté résistante à la lyse, comme marqueur pour la reconstruction des environnements passés. Premièrement, l'efficacité de la nouvelle méthode développée pour l'isolation des spores a été évaluée en comparant la communauté totale et la communauté résistante à la lyse. La composition de ces communautés a été comparée dans des échantillons qui ont été soumis ou non au traitement de séparation des spores. Les résultats ont montré que la communauté résistante à la lyse possède une signature unique par rapport à la communauté totale. Il est intéressant de noter que divers genres jusque-là considérés comme non-sporulants ont été trouvés en forte abondance dans la communauté résistante à la lyse, mais ne faisaient pas partie des genres les plus abondants de la communauté totale. Cela démontre une capacité à résister au traitement de séparation des spores et suggère fortement la capacité de ces groupes à produire une structure résistante à la lyse. Deuxièmement, la méthode de séparation a été utilisée pour étudier la diversité, la distribution et la structure de la communauté des organismes potentiellement sporulants dans l'environnement. Dans ce but, des données ont été obtenues à partir de quatre sites d'étude distincts. Les résultats de notre analyse ont révélé une diversité insoupçonnée d'organismes dans la communauté résistante à la lyse. Cette dernière a également présenté un pattern de distribution géographique, remettant en question une hypothétique distribution cosmopolite des bactéries sporulantes. Les résultats de cette étude suggèrent que la capacité de former des spores ou des structures cellulaires résistantes similaires est plus répandue qu'on ne le pensait jusque-là. Dans

une deuxième étude, l'application de ces nouvelles méthodes a été testée pour reconstruire l'histoire du lac Liambezi, un lac éphémère situé en Namibie. En utilisant une approche multidisciplinaire incluant l'utilisation d'ADN bactérien en complément d'analyses géochimiques et sédimentologiques, l'évolution du climat au cours des 5500 dernières années a été reconstruit. Cela a mis en évidence une alternance de périodes sèches et humides, et des changements dans le régime hydrologique du lac (de marais à lac). L'ADN a été isolé à la fois de la fraction totale et de la fraction résistante à la lyse de la communauté, les deux reflétant des changements de conditions environnementales, et démontrant leur pertinence pour les études paléoécologiques. Fait intéressant, dans certains cas, des conditions environnementales supposées, des processus biologiques ou des événements extrêmes, n'ont été observés que dans une fraction de la communauté, ce qui met en évidence la complémentarité et l'intérêt d'étudier les deux fractions de la communauté. De plus, l'analyse de la communauté bactérienne et de populations spécifiques a permis d'élaborer un modèle d'âge cohérent reliant les trois carottes de sédiments étudiées. Cette analyse suggère une activité hydrothermale et un cycle du soufre dans le lac. Les résultats de cette étude ont démontré le potentiel de l'utilisation de l'ADN bactérien (total et/ou spores) pour identifier des changements et la variabilité des conditions environnementales, ainsi que l'intérêt d'une approche multidisciplinaire pour reconstruire l'histoire des écosystèmes.

La deuxième partie de cette thèse visait à évaluer l'utilisation possible de ces nouvelles méthodes pour étudier l'abondance des gènes de résistance aux antibiotiques (ARG) dans l'environnement. Depuis la découverte de la pénicilline par Fleming en 1928 et le développement d'antibiotiques à usage médical à partir des années 1940, l'utilisation généralisée d'antibiotiques a créé une pression de sélection qui a contribué à l'émergence de bactéries résistantes aux antibiotiques (ARB) et de bactéries multi-résistantes (MRB). La multiplication de ces organismes représente une menace réelle pour la santé humaine à l'avenir. Longtemps ignoré, ce problème est désormais considéré comme urgent. Bien que la distribution et la fréquence des ARG dans l'environnement soient largement méconnues, il est clair que les activités humaines ont un impact sur leur abondance. Dans cette partie de la thèse, l'utilisation d'ADN extrait de cellules bactériennes résistantes à la lyse pour tracer les ARG dans l'environnement a été évaluée. Dans une première étude, l'accumulation d'ARG au cours du temps a été étudiée, dans les communautés totale et résistante à la lyse, dans les sédiments du lac Léman (CH). Les résultats ont montré que deux ARG sélectionnés (*tet*(W) et *sul*1) pouvaient être détectés à la fois dans la fraction résistante à la lyse et dans la fraction totale de la communauté. Les deux gènes ont été trouvés à une fréquence (copies/ng d'ADN) plus élevée dans la communauté résistante à la lyse de la communauté par rapport à la communauté totale, suggérant que la fraction résistante à la lyse était enrichie en ARG. Les profils d'accumulation de ces deux ARG ont montré une corrélation avec l'utilisation historique de leur antibiotique relatif. En étudiant la relation entre l'accumulation d'ARG et la composition de la communauté bactérienne, chaque gène a montré être corrélé à un groupe taxonomique différent. Alors que *tet*(W) était principalement corrélé à un changement dans l'abondance relative des Firmicutes, *sul*1 était corrélé à un groupe d'organismes plus diversifié, suggérant que les deux ARG sont distribués différemment entre les taxons bactériens. Ces résultats montrent que des structures assimilables à des spores peuvent être utilisées pour retracer l'effet de l'utilisation historique d'antibiotiques sur l'abondance des résistances. Dans une deuxième étude, l'impact des rejets d'eaux usées sur la dispersion environnementale des ARG associés à la communauté résistante à la lyse a été étudié dans les sédiments de la baie de Vidy (CH). L'usine de traitement des eaux usées (STEP) a été identifiée comme une source d'ARG. Les deux ARG étudiés (*tet*(W) and *sul*1) ont été détectés dans tous les échantillons, leur abondance/fréquence diminuant avec la distance jusqu'à l'exutoire de la station d'épuration. Les niveaux d'ARG étaient corrélés à d'autres indicateurs de rejet d'eaux usées, tels que le C_{org}, le N_{tot} et l'ADN. Les deux ARG se sont révélés être différemment enrichis dans la communauté

résistante à la lyse. De manière similaire à ce qui avait été observé dans la première étude, la fréquence de *tet*(W) était principalement corrélée à l'abondance relative de genres appartenant aux Firmicutes (*Clostridium* et *Rminococcus*), et la fréquence de *sul*1 était corrélée avec un large spectre taxonomique d'organismes. La forte abondance relative de *Clostridium* spp., couplée à sa corrélation avec la fréquence de *tet*(W), suggère que les membres de ce genre pourraient être un vecteur potentiel de dissémination de *tet*(W).

Ces deux études constituent la première preuve d'une possible détection d'ARG dans des spores ou des structures résistantes à la lyse. L'ADN extrait de ces structures résilientes a démontré être un bon marqueur pour évaluer la dispersion et l'accumulation d'ARG au cours du temps dans des échantillons environnementaux. Compte tenu de leur forte capacité de survie et de leur propension à la dispersion, cette fraction de la communauté résistante à la lyse pourrait recevoir une attention particulière à l'avenir, pour une meilleure compréhension de son rôle dans le devenir des ARG, et pour aider à mettre en œuvre des stratégies d'utilisation appropriées pour lutter contre la dissémination des résistances aux antibiotiques et améliorer leur élimination lors du traitement des déchets.

Mots-clés: spores, résistant(e) à la lyse, paléoécologie, résistance aux antibiotiques, communauté bactérienne, sédiment.

1 Introduction

1.1 Paleoecology

1.1.1 Paleoecology

Paleoecology is the study of past ecosystems using sedimentary records or other natural archives (sediment, rock, coal, petroleum, ice core, speleothems). It aims to establish the relationship between ancient life forms and their environment (Willard & Cronin, 2007). Depending on the rate of deposition and the size of the sediment records, studies can be carried out on multiple time scales. Analyzing the chemical, isotopic and/or biological composition of sediments can provide keys for the reconstruction of ecosystem history and studying their response to environmental change. Likewise analyzing the structure of biological communities and its change along a sediment core allows to address specific questions regarding the variability/stability of communities over time, the effect of disturbance on these communities and/or capacity of resilience of an ecosystem (Gorham et al., 2001).

From a more "modern times" perspective, growing concerns about climate change, degradation of natural environments and/or depletion of natural resources call for the need of a better understanding of ecosystem functioning and impact of human activities on ecosystems health. Nowadays, most aquatic ecosystems such as lakes, river deltas or estuaries, among others, are under considerable anthropogenic pressure, due to activities such as extensive agriculture, tourism, and industry. These ecosystems have high ecological and economical values. It is thus of prior importance to study the relationship between timeline land-usage and the ecosystem health.

As an example of the need for studying ecosystem history of aquatic ecosystems, we can mention the "not-so-old" European Water Framework Directive (EU 2000), which fixed as an objective to restore the "natural ecological status" of all European lakes by 2015. However, this "natural ecological status" is simply unknown and must be clearly defined. The effect of disturbance versus natural fluctuation has to be established, as well as the capacity of resilience of the ecosystems. Only such knowledge could allow taking appropriate measures for the protection of these ecosystems (Junier, Vennemann & Ariztegui, 2014).

1.1.2 Biological proxies

With its outlet function, lakes and their sediments reflect the general land-usage of a whole catchment and are appropriate for a better understanding of the effect of human activity on ecosystem health. Sediments constitute a natural archive of past environments and its chemical and biological composition provides valuable information for the reconstruction of ecosystem history. In addition to the mineralogical, chemical and isotopic composition of sediments, which have been used successfully for reconstruction of past environments, a wide variety of biological proxies (including entire organisms, remains, chemical compounds, or changes in isotopic composition linked to biological activity) are commonly used for studies in paleoecology (see Gorham et al. 2001; Meyers 2003; Castañeda & Schouten 2011 for reviews). Examples of these proxies include not exhaustively pollen grains (Girardclos et al., 2005; Waldmann et al., 2014; Matthias, Semmler & Giesecke, 2015; Stolze, 2015), siliceous and calcareous microfossils (testae such as diatoms, ostracods) (Decrouy, Vennemann & Ariztegui, 2011a,b; Decrouy & Vennemann, 2013; Waldmann et al., 2014), lipids (Niemann et al., 2012) or fish scales (Gerdeaux & Perga, 2006). All these structures/compounds have the particularity to remain unaltered in sediments for extensive periods of time, which makes them adapted for paleoecology.

The use of structures associated to bacteria for paleoecological studies is of particular interest since bacteria are the most abundant organisms on earth and have a considerable cumulative mass in lakes (water column and sediment). Moreover, bacteria with their remarkable phylogenetic and metabolic diversity, and high dispersal rates, have colonized all ecosystems (Nealson, 1997). To date, most studies using bacteria-linked structures have focused on fossil pigments (Gorham et al., 2001; Dreßler et al., 2007). More recently, technological advances in the field of metagenomics allowed the study of microbial communities and evaluation of microbial diversity using "ancient" or "fossil" DNA as a possible proxy (Coolen & Gibson, 2009; Boere et al., 2011; Fernandez-Carazo et al., 2013; Pansu et al., 2015). However the use of DNA-dependent methods for the study of bacterial communities in paleoecology is problematic as the preservation of DNA in sediments is taxon-dependent and influenced by multiple biotic and abiotic factors (Boere et al. 2011 and references therein). Moreover, the possible modification of the community structure within the sediment could lead to misinterpretations.

Bacterial resting states (spores) might allow overpassing these limitations. These highly resistant structures tolerate harsh environmental conditions, surviving in a dormant state for long time. The use of such structures has been examined and proposed as an alternative to total DNA-based analyses (see Renberg & Nilsson 1992 for a review). In this thesis, the use of endospore-forming bacteria as a biological proxy for the reconstruction of lakes history will be explored using molecular tools recently developed.

1.1.3 Endospore-forming bacteria

The capacity to form spore-like structures is spread among bacteria and has been reported for different groups such as Actinobacteria, Gram-positive filamentous bacteria (Ensign, 1978; Chater & Chandra, 2006); Myxobacteria, Gram-negative Deltaproteobacteria (Strauch & Hoch, 1992; Thomas et al., 2008); Cyanobacteria, aerobic phototrophic bacteria (Adams & Duggan, 1999); and Firmicutes, Gram-positive low G+C content bacteria (Onyenwoke et al., 2004). The latter is particularly interesting and might be an appropriate candidate for the role of proxy:

- Among the different organism able to produce spore-like structures, the formation of heat-resistant endospores has only been reported for Firmicutes (Galperin et al. 2012; Abecasis et al. 2013; Wunderlin et al. 2014)
- In culture collections, Firmicutes represent the second most abundant bacterial phylum known (Klenk & Göker, 2010), providing a precious source of metabolic information/knowledge.
- The broad diversity of metabolisms found in Firmicutes allows this clade to colonize a wide range of habitats. Among Firmicutes we found aerobic, facultative and strictly anaerobic representatives, including non-exhaustively sulfate-reducers, sulfide-oxidizer, phototrophs or homoacetogens (Collins et al., 1994; Norris et al., 1996; Nicholson et al., 2000; Schleifer, 2009).
- Firmicutes are considered as ubiquitous, due to the advantage given by sporulation in terms of dispersal and survival to environmental disturbances, and their broad diversity of metabolisms (Nicholson et al., 2000; Nicholson, 2002; Mandic-Mulec & Prosser, 2011).

Endospore-forming bacteria are a paraphyletic group of Gram-positive bacteria within the Firmicutes (Onyenwoke et al., 2004; De Hoon, Eichenberger & Vitkup, 2010). Firmicutes are divided in three classes: *Bacilli, Clostridia* and *Erysipelotrichi* (Schleifer, 2009), all known spore-formers belong to the first two classes. Bacilli are mostly aerobic while *Clostridia* are mostly anaerobic. In Firmicutes the spore is formed inside a "mother cell", hence the name of "endospore" (Driks, 2002). Endospores are

highly specialized structures that can resist to desiccation, high and low temperature, and radiation (Nicholson et al. 2000 and references therein).

1.1.4 Culture-dependent studies and quantification of dipicolinic acid

Most knowledge about endospore-formers comes from culture-dependent studies made in laboratory (Nicholson, 2002). Spore isolation and revival is an old technique (Bartholomew & Paik, 1966; Renberg & Nilsson, 1992). However, culture-dependent studies are known to be biased, due to the small fraction of organisms amenable to cultivation (Amann, Ludwig & Schleifer, 1995). Revival of endospores has been used in paleoecological studies for the reconstruction of past environments or to demonstrate the dispersal ability of endospores. *Clostridum perfringens* or *Thermoactinomyces vulgaris* have been shown to serve as a paleoindicator for sewage pollution or agricultural activity, respectively (Renberg & Nilsson 1992 and references therein). Several studies reported the isolation of endospores from thermophilic strains in cold marine sediments, demonstrating the dispersal of endospores over thousands of years (Bartholomew & Paik, 1966; Hubert, 2009; de Rezende et al., 2013; Müller et al., 2014). Studying a sediment core of 7 m representing the past 13'000 years in Lake Constance, Rothfuss et al. (1997) showed that all viable heterotrophic bacteria under 25 cm were in the form of endospores. Endospores abundance decreased exponentially until 6 m (~8'900 years old.), below what it was not detectable. Endospores have been reactivated from sediments as old as ~9'000 years old (Nilsson & Renberg, 1990; Rothfuss, Bender & Conrad, 1997). More "controversial" studies reported the revival of *Bacillus* endospores from 25- to 40-million-year-old (Cano & Borucki, 1995) and 250 million-year-old records (Vreeland, Rosenzweig & Powers, 2000).

Alternatively, most culture-independent studies are based on the detection/quantification of dipicolinic acid (DPA), a biomarker specific to endospores (Fichtel et al., 2007). Different methods have been developed for the extraction and quantification of DPA, with detection limits ranging from 10^8 endospores/g of sediment to 10^3 endospores/ml (attention to the unit of measurement; see Bueche et al. 2013 for a review). These methods have three main disadvantages:

- Consider only the spore fraction but not the vegetative cell fraction.
- Interferences can occur in the measurement due to the presence of humic acid or organophosphates, commonly present in sediments (Fichtel et al., 2007).
- Destructive method, spores have to be destroyed to release dipicolinic acid.

In sediments of several millions of years, the estimation of endospores abundance shown that endospores were as abundant as vegetative cells (Lomstein et al., 2012). A study in marine sediments from the North Sea revealed that endospores represented up to 3% of the total bacterial community (Fichtel et al., 2007) while a subsequent study (Fichtel et al., 2008) reported that the contribution of endospores in total cells count could reach 10% in deeper sediment, whereas it was only 1% in the upper part of the core (first 50 cm, 3 cores up to 5.5 m). Moreover 10% is probably underestimated due the uncomplete recovery of DPA in gray mud from which the sample originated. Results of this study also demonstrated that endospores abundance in marine sediments strongly depends on lithology: higher in black mud, endospores number decreased gradually with increasing proportion of sand. Although endospores abundance was irregular in small scale, it was relatively constant with depth. Brandes Ammann et al. (2011) also revealed a relation between endospores abundance and soil type, depth, and C/N ratio. The endospores abundance was the highest in samples from grasslands (agriculturally used land: pasture, meadow) compared to forests and fluvial sediments had the lowest abundance.

1.1.5 Molecular tools

Although endospore-forming Firmicutes have been evaluated as paleoecological proxies and used in previous studies, molecular methods designed specifically to investigate this group were until recently unavailable. Some specific primers have been designed for targeting specific groups, for example *Bacillus* (Garbeva, Van Veen & Van Elsas, 2003) and *Paenibacillus* (da Silva et al., 2003), but no primers existed for the whole community of endospore-formers. Sporulation is a complex mechanism involving many regulatory genes. Interestingly, several studies have identified common genetic elements involved in endospore formation. In particular a common set of genes responsible for the formation of a minimal sporulation core has been identified (Galperin et al., 2012; Abecasis et al., 2013). These findings had not led to the development of molecular methods for the study of endospore-formers, but provided precious information for the development of a molecular probe targeting the endospore-forming bacteria.

With the perspective of using molecular tools, another advantage to focus on a subset of the whole community is that it improves the "sequencing effort", offering a higher coverage and resolution for the targeted group (Suenaga, 2012). Effectively, the sequencing of a whole complex community is problematic and does not reflect the "true" diversity of the community (Gilbert & Dupont, 2011). Moreover, the high complexity of bacterial communities makes it very difficult, if not impossible, the analysis of trends in the change of these communities (Wunderlin et al. 2014). Another argument for the need of a DNA extraction method specific to endospore-formers is the probable underrepresentation of spore-formers in "traditional" genomic studies. In their "phylogenetic assessment of microbial communities in diverse environments", von Mering et al. (2007) found that spore-formers were surprisingly scarce. These results may be explained by the difficulty to extract DNA from highly-resistant structures that are spores, with protocols mostly adapted to vegetative cells (Wunderlin et al. 2014; Filippidou et al. 2015). All of these prompted the development of new molecular methods, specifically developed for the study of endospore-forming bacteria. A gap that has been filled these last several years, with the development of a series of molecular tools dedicated to the study of endospore-forming bacteria.

1.1.6 New molecular tools for the study of endospore-forming bacteria

Identification of a genetic marker (Wunderlin et al., 2013)

First, a genetic marker specific for endospore-forming bacteria has been identified. Within a set of six genes involved in the formation of the sporulation core, *spo0A* gene was selected and specific primers have been developed. This marker, the stage 0 sporulation gene A (*spo0A*), is the master regulator for the sporulation pathway. It has only been reported to be present in Firmicutes (Galperin et al., 2012; Abecasis et al., 2013). Phylogenetical analysis based on *spo0A* sequences were consistent with analyses based on the core genes and 16S rRNA gene. Separation of the *Bacillus subtilis* and *Bacillus cereus* clades, as suggested by Bhandari et al. (2013) was also supported. Moreover, *spo0A* sequences exhibit two conserved and a highly variable region. Another interesting aspect is that *Spo0A* is one of the most-studied sporulation genes, and is often automatically annotated. Thus, the number of *spo0A* sequences in databases increases rapidly. Based on the alignment of *spo0A* sequences from 27 known genomes, a pair of degenerated primers was selected, within a set of 7 forward and 10 reverse primers tested in all combinations. The product amplified by this pair of primers is a 602 bp sequence. This pair of primers was then tested for validation with a selection of 53 pure cultures, including mostly members from the class *Bacilli* from different genera, three members of *Clostridia* and non-endosporulating

strains from both inside and outside the Firmicutes. The primers showed a good coverage even if the amplification was negative for *Alicyclobacillus* and *Sulfobacillus*. No amplification occurred with non-endosporulating strains.

DNA extraction method (Wunderlin et al., 2013)

A DNA extraction method was developed, adapted to highly-resistant structures such as spores and allowing the release of DNA from endospores. This DNA extraction protocol is based on the use of the "FastDNA Spin kit for soil" (MP Biomedicals, Solon, OH, USA). Tests were conducted on both cultures of vegetative cells and spores. Application of three repetitive extractions (bead beating) for the lysis of spores led to higher DNA yields, particularly for spores, and was retained as the best compromise between the overall DNA yield and the quality of the DNA extract. Time and cost were also taken into account.

For environmental samples, an indirect extraction method was developed with the aim of separating the biomass from sediment particles. With this indirect method, DNA yields are lower (DNA yield does not only depend on cell lysis), but gene abundance (16S rRNA and *spo0A*) was higher. This inverse correlation suggests a better quality of DNA extract with the indirect method, probably due to co-extraction of inhibitors with direct extraction.

Quantification method by qPCR (Bueche et al., 2013)

A method for quantification of endospore-forming bacteria had also been developed. The same specific marker (*spo0A*) and an approach by quantitative PCR have been selected. The same targeted gene as for metagenomics analyses (*spo0A*) was selected, because of the consistence of the phylogenies obtained from both *spo0A* and 16S rRNA. The primer design was based on a multivariate analysis, allowing for the finding of the most appropriate conserved regions. Eleven sites were evaluated as potential primer site, for amplicons of approximately 150 bp. Two sets of primers were selected and tested for 16 pure cultures from different genera of endospore-forming Firmicutes (*Bacillus, Paenibacillus, Brevibacillus, Geobacillus, Alicyclobacillus, Sulfobacillus, Clostridium* and *Desulfotomaculum*) and environmental samples. Primers set 1 mostly amplified *Bacilli*, whereas set 2 showed to be more universal. Only *Sulfobacillus* and *Desulfotomaculum* did not exhibit amplification. The detection limit was ~10^4 cells or spores per g of material.

Use of endospore-forming bacteria as a proxy (Wunderlin et al. 2014)

The different methods presented above (qPCR, DNA extraction, *spo0A* amplicon sequencing) have been used to assess the possible use of endospore-forming bacteria for paleoecological reconstructions. A sediment core of ~1 meter representing ~100 years (from 1921 to 2010) was retrieved form Lake Geneva, dated and subjected to different chemical and biological analyses. Bio-informatics tools for the analyses of pyrosequencing results were also developed and are recurrently upgraded. These tools notably allowed the phylogenetical affiliation of *spo0A* amplicons and the reconstruction of endospore-forming communities (Junier et al., 2015).

Results indicated that DNA and 16S rRNA gene abundances decrease exponentially with depth whereas *spo0A* was relatively constant, demonstrating the potential for conservation of DNA from endospore-forming bacteria in sediments. Importantly the majority of endospore-forming bacteria might be

autochthonous, contrarily to what have been related in other studies (Bartholomew & Paik, 1966; Robles et al., 2000; Hubert et al., 2010). A correlation between eutrophication and change in the endospore-forming community was demonstrated. Before 1961, endospore-forming community was dominated by aerobic groups (*Bacillus*) and facultative anaerobes (*Paenibacillus*). Between 1961 and 1987, anaerobic groups were dominant (*Clostridium*, *Desulfitobacterium*). And from 1997 community was again dominated by *Bacillus*. In consequence, between 1961 and 1987, bacterial community composition is linked with a decrease in oxygen availability. The shift in the community between 1961 and 1997 was connected with low C/N values and higher TOC and Fe/Mn, all indicating hypoxia/anoxia and reflecting eutrophication. In upper sediments a trend to the recovery (or resilience) of the community was suggested, but delayed compared to water nutrients.

Method for the isolation of endospores (Wunderlin et al. 2014)

Another key point of our research is the question of the active versus inactive fraction of the endospore-formers community. The separation of endospores fraction from the total community of endospores-formers is of great interest since the ecological role of each fraction is not the same. The endospore fraction can be considered as a "seed bank", a reservoir of dormant organisms able to reactivate in case of favorable environmental change. This constitutes a great advantage to these organisms in terms of adaptability. However, in case of paleoecological studies, it is not clear whether this "seed bank" can be used as a proxy. If endospore-forming bacteria were not active, they may not be representative of the environmental conditions at the time of deposition. This question is of main importance as one of the advantages of using endospore-forming bacteria is the possible use these high resistant structures that can be preserved in sediments.

In order to study the inactive fraction of endospore-forming bacteria, a method for the physical isolation of endospores was developed in our laboratory (Wunderlin et al. 2014). For testing the efficiency of this method, treated and untreated environmental samples (Lake Geneva and Lake Baikal) were compared based on the amplification of 16S rRNA gene. The endospores isolation method of isolation allowed enrichment of targeted Firmicutes from 8-19% to 83.9-90.6% of the total community, proving the efficiency of the method. Other spores-like microbial group represented less than 2%. Finally, members from the two main classes of Firmicutes (*Bacilli* and *Clostridia*) were detected. This new method for separation of spores was used for a second study on Lake Geneva. Based on the results of the previous one, additional data had been collected: sequencing effort (*spo0A* amplification) has been expanded to supplementary depths and some of them have been selected for the treatment for separation of spores. Although analysis and interpretation of the data are still in process, first results suggest a correlation between changes in endospore-forming bacteria communities and different environmental events. In addition to the eutrophication of the lake between 1960 and 1990 previously mentioned and confirmed here, other events could be highlighted, such as low temperature registered in 1929, increasing use of fossil fuels (coal) in the 1950s or terrestrial organic matter input in sample from 1997.

The development of this innovative method represents a step forward for the study of this group of bacteria, in terms of diversity, ecology and metabolic potential. Composition of the communities resulting from the endospores treatment allows determining the "seed bank" component of the communities, isolating the endospores (inactive) from the vegetative cells. Comparison of active versus inactive fraction of endospore-forming bacteria may allow refining the interpretation and identification of perturbation events, and better understanding the response of communities to these environmental changes.

1.2 Antibiotic resistance genes

1.2.1 Antibiotic resistance genes in the environment

The second part of the thesis aimed at enlarging the use of endospores to another subject of great interest and concern: the dispersal and persistence of antibiotics resistance genes (ARG) in the environment. The development of antibiotics for treatment of human diseases since the 1940s' is probably one of the most important progress in the medical field of the last century (Marti, Variatza & Balcazar, 2014). Nowadays, several hundred different antibiotics are used in human and veterinary medicine but data on their real use are scarce (Kümmerer, 2009). Despite the benefits that this represents in the field of medicine, it is now admitted that there is a link between the extensive use of antibiotics and the emergence of highly resistant and multi-resistant bacteria (MRB) (Marti et al. 2014).

From a historical/biological point of view, antibiotics are natural compounds produced by organisms as a chemical weapon for inhibiting or killing other organisms in a context of competition. Resistance to these antibiotics is an ancestral feature giving the organisms that own it, the immunity against these chemical agents. Such resistance has existed long before the use by humans of antibiotics for medical purposes. ARG have been detected in ancient environments considered free of human influence: D'Costa et al. (2011) reported the detection of ARG (resistance genes for b-lactam, tetracycline and glycopeptide antibiotics) in permafrost from Beringian (30'000 years old). In that study they showed that ancient and recent genes were phylogenetically closely related, demonstrating that structure and function could be conserved over millennia. Even older, 14 different ARG have been detected in isolates from Lechuguilla Cave (Bhullar et al., 2012). The culture collection was obtained from a part of the cave that was isolated for the past 4 million years, with no source of water from the surface. Again, conservation of structure and function has been demonstrated.

In modern environments, the abundance and diversity of ARG in natural ecosystems at global scale is widely unknown. Despite the real threat for public health represented by ARG, relatively few efforts have been made for monitoring their occurrence and expansion in environment (Marti, Variatza & Balcazar, 2014) and there is a lack of data about their natural background in natural ecosystems (Czekalski et al., 2012). The prevalence and source of ARG in the environment is still controversial (Bhullar et al., 2012). It has been demonstrated that horizontal gene transfer (HGT) is the main mechanism by which bacteria can exchange ARG. Such transfer can occur between both environmental strains, pathogens and also phylogenetically distant organisms (Pruden et al., 2006). This makes the discrimination between natural-acquired resistance and human-induced resistance difficult.

However human activities are pointed as a major source of ARG, mainly due to runoffs from agricultural/industrial activities and discharge of treated or untreated wastewater (Marti, Variatza & Balcazar, 2014), and "The European council concluded in 1998 that there was a relationship between the consumption of antimicrobial compounds and the prevalence of antibiotic-resistant bacteria" (Perry, Westman & Wright, 2014). Although resistance to antibiotics is ancient, there is no doubt the selection pressure caused by the extensive use of antibiotics affects their mobility and abundance. The ARG pool in environment represents a real threat for human health, with a risk of transmission of ARG from environmental organisms to pathogens and inversely. D'Costa (2006) notably revealed an unsuspected diversity and abundance of ARG in soil microbiome, highlighting the high potential reservoir function of environment.

The problematic is highly complex and involves many actors and factors: pathogens and environmental bacteria, bacterial density, anthropogenic activities, environmental compartments, mechanisms of gene transfer, selection and co-selection processes with other pollutants and environmental conditions. Highly resistant bacteria are assumed to emerge mostly from clinical and animal environments due to the extensive use of antibiotics (Baquero, Martínez & Cantón, 2008; Czekalski et al., 2012). These antibiotic resistant bacteria (ARB) are notably released in natural ecosystems via sewage effluent, hospital wastewater, agricultural/farming runoff and/or aquaculture discharge (Taylor, Verner-Jeffreys & Baker-Austin, 2011; Marti, Variatza & Balcazar, 2014). Once release in aquatic ecosystems ARB/ARG can also be dispersed in the environment, with a potential to be reintroduced in the "human compartment" via drink water pumping systems, fish keeping or recreational activities, for example. They might also accumulate in sediments or biofilms. Sediments are the main sink for ARG (and other pollutants) in aquatic systems and provide a propitious environment for ARG transfer, constituting both a reservoir for ARG and a reactor for their evolution. In these aquatic environments, bacteria from human and animal microbiomes, potentially including several pathogens, co-exist with environmental bacteria. Whereas the overall abundance and diversity of ARG in the environment is widely unknown, aquatic ecosystems are recognized as a huge potential ARG reservoir. Thus, this mixture might allow gene transfer leading to both increasing the ARG pool in environment and transmission of ARG to human/animal pathogens.

Moreover, the extensive use of antibiotics induces a selection in all compartments of the ARB/ARG pathway. Effectively this selection pressure can occur in the human or animal microbiomes, but also in wastewater treatment plants (WWTP), soils, biofilms and sediments. Baquero et al. (2008) illustrates this complex system, using the term of "genetic reactors" to describe the different compartments involved in the spread and evolution of ARG (Figure 1). Not only antibiotics induce a selection pressure on ARG and other pollutants can also impact the evolution of ARG. Gillings & Stokes (2012) suggest that mutation and transfer rates are influenced by the "fitness cost", in other words environmental stress promotes mutation and gene transfer and leading to an advantage in terms of evolution. Moreover, WWTP also release large amount of trace metal, which are assumed to co-select for ARG (Berg et al., 2010). Sediments impacted by WWTP discharge may represent a "perfect" environment for evolution and transfer of ARG.

Here we propose to evaluate the potential of using endospores for tracking ARG in sediments. The highly resistant structure of endospores allows their preservation in sediments, contrarily to vegetative cells and free DNA. Endospores might be appropriate candidates for the study of ARG accumulation over time. Moreover, recent studies reported a correlation between Firmicutes abundance and trace metal concentration in contaminated sediments (Bueche, 2014), and suggested possible co-transport and/or co-selection mechanisms. We propose to explore further this possible link and the role of endospores in dissemination of ARG.

1.2.2 Aquatic ecosystems and ARG

Aquatic ecosystems and lakes in particular are sensitive zone having a central role in the spread and accumulation of ARG in environment. On the one hand they are exposed to concomitant release of antibiotics and ARB/MRB from anthropogenic source (wastewater, agricultural runoffs, Figure 2), and on the other, they have a reservoir function for ARG (Czekalski et al., 2012; Marti, Variatza & Balcazar, 2014; Devarajan et al., 2015). Since many lakes are source for drinking water and/or recreational areas, they call for the need of program surveys and assessment of the risks for human health.

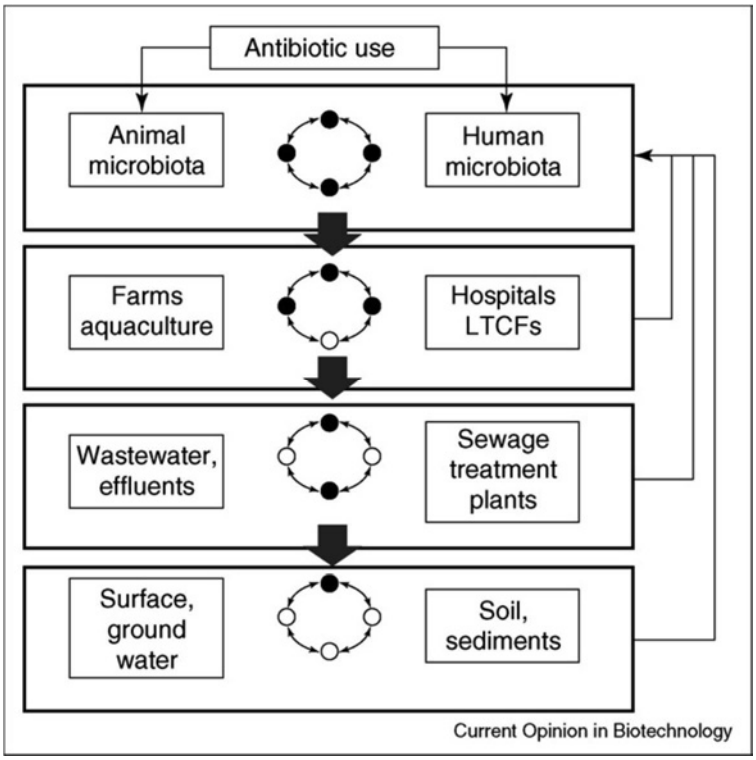

Figure 1: Diagram illustrating the "four genetic reactor in antibiotic resistance", responsible for the dispersal and evolution of ARG (taken from Baquero et al. 2008). The crossing from one reactor to another is illustrated by the large black arrows, leading to the progressive mixing of human/animal-associated bacteria (black circles) with environmental bacteria (white circles), and downstream reintroduction to human and animal environments (back arrows). LTCFs stand for long-term care facilities.

Sediments and biofilms have been identified as hotspots for accumulation and transfer of ARG. In aquatic ecosystems, bacteria often organizes in biofilms, which are highly organized structures composed of dense bacterial communities. Such structures may promote HGT within bacterial community (see Taylor et al. 2011; Marti et al. 2014 for reviews). Evaluating the prevalence of ARG in biofilms from diverse locations, including WWTP influent, effluent and drinking water systems, Schwarz (2003) detected resistance genes (*vanA* and *ampC*) not only in biofilms from wastewater system of a hospital, but also in biofilms from drinking water system. Moreover, *vanA* was detected in the biofilms from drinking water system in absence of *Enterococci*, suggesting a possible gene transfer from pathogens to autochthonous bacteria and highlighting the potential risk for human health to reintroduce resistant organisms through drinking water distribution. Another study investigating the fate and persistence of ARG in aquatic environments showed that some ARG (*tet*(W)) tend to migrate easily from water column to biofilm, which thus constitute a potential long-terms reservoir for ARG (Engemann et al., 2008).

Taylor et al. (2011) describe sediments as “an environmental matrix within which HGT can occur”. In aquatic ecosystems they represent the main sink for both ARG, antibiotics and other pollutant such as trace metals. Coupled to a high bacterial density and diversity, this creates favorable conditions promoting HGT and (co-)selection of ARG, and thus having a significant role on the spread, persistence and evolution of ARG (Taylor, Verner-Jeffreys & Baker-Austin, 2011; Marti, Variatza & Balcazar, 2014). The accumulation of mobile genetic elements (MGE, often associated with antibiotic resistance), respectively plasmids and integrons, have been reported for contaminated sediments. For instance, Cummings et al. (2011) detected five different “plasmid-mediated quinolone resistance (PMQR)

determinants" in an urban coastal wetlands impacted by sewage, against (and only) three in another one free of impact. Kristiansson et al. (2011) reported the overrepresentation of MGE in river sediments exposed to antibiotics. Studies comparing ARG/MRB abundance in sediments and its upper water column showed their abundance was up to three orders of magnitude higher in sediments (Chen et al., 2013). Again, both studies point at the fact that ARG concentration in sediments depends on the degree of anthropogenic impact. Comparing resistome from marine sediments and human pathogens using metagenomics approach, Yang et al. (2013) found genes sharing high similarity between both, suggesting marine sediment might play an important role in ARG transfer. Not only these potential hotspots (biofilm, sediments) of bacterial activity could promote gene transfer, but some organisms or biological structures commonly found in aquatic ecosystems (ciliates, and chitin constituting the shelves of crustacean, fish intestinal system) are also favorable to gene exchange (see Marti et al. 2014 for a review). All these studies highlight the complexity of the problem and the lack of knowledge in this field, and the need for a better understanding of the numerous pathways involving the transmission and dissemination of ARG in the environment.

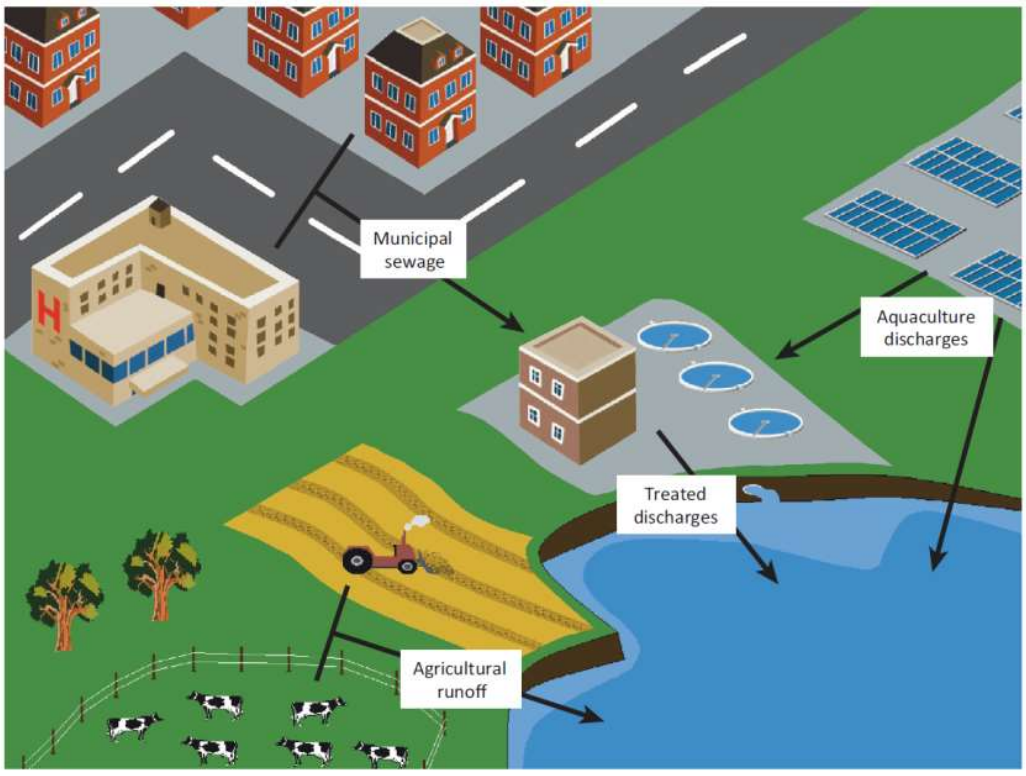

Figure 2: Main source of ARG/ARB in environment (taken from Marti et al. 2014).

1.2.3 Wastewater

Several studies have pointed out WWTP as a major source of ARG in environment (Baquero, Martínez & Cantón, 2008; Marti, Variatza & Balcazar, 2014). Many WWTP not only collect wastewater from domestic/urban sources, but also from hospitals and other clinics, which release considerable amounts of pathogens, ARB and ARG. It is assumed that MRB results from the extensive use of antibiotics and emerge mainly from hospitals (Baquero, Martínez & Cantón, 2008; Czekalski et al., 2012), highlighting the primary importance of the removal efficiency of the wastewater treatment in WWTP. Moreover it has suggested that WWTP may promote HGT, due to favorable conditions (high bacterial densities,

high oxygen and nutrient concentrations) and the continuous co-occurrence of both ARB and antibiotics (Rizzo et al., 2013). As an example of the considerable amount of ARG found in WWTP, a metagenomics study allowed the detection of respectively 140 and 123 different ARG in the activated sludge and the effluent of a WWTP (Szczepanowski et al., 2009).

Results of the studies about the effect of WWTP on ARG abundance are contrasted. Whereas some studies reported a general decrease of ARG after wastewater treatment, other reported an inverse effect (Czekalski et al. 2012 and references therein). In that case, the increase of ARG is attributed to selective effect of WWTP. We also have to keep in mind that most studies have been made using culture-dependent methods. In more recent studies based on PCR-dependent methods and qPCR quantification, Xu et al. (2015) concluded that no significant change in diversity and abundance of ARG occurred through wastewater treatment in a Chinese WWTP. This contrasts with results obtained by Rodriguez-Mozaz et al. (2015) who reported a general decrease of ARG after wastewater treatment. However, some of the ARG tested were still present in WWTP effluent. In both cases the removal was different depending on the class of ARG. More interestingly, even in the case of a decrease of total ARG abundance (copies number), a general increase of relative abundance (ARG/16S rRNA) was reported. In addition, correlations between antibiotics concentrations and their associated ARG abundance was reported. This might suggest selective processes and/or HGT occurring during wastewater treatment. It has been shown that ARG/ARB could pass through wastewater treatment even with elaborate treatment and disinfection (LaPara et al., 2011). Comparing wastewater from five WWPTs at different stages of their treatment, Munir et al. (2011) reported a high variability in ARG removal depending on the type of wastewater treatment. Even if a general decrease of ARG was noticed, “Membrane Biological Reactor” has shown to reduce the prevalence of ARG up to three orders of magnitude more than conventional treatment.

In summary and considering these contradictory results we can assume that in most case ARG/ARB are not completely eliminated from wastewater. Whereas some ARG may be removed from wastewater, on the contrary others might be enriched, due to the favorable environment provided in WWTP and depending on the type of treatment, the concentrations of associated antibiotics and probably other factors that still need to be determinate. Despite a partial removal, concentration of ARG in the effluent of WWTP are too high, and reach level possibly impacting environment and bacterial communities (Czekalski et al., 2012; Rodriguez-Mozaz et al., 2015). WWTP not only fail to eliminate ARG/ARB from wastewater, they might promote selective processes and HGT, thus having an important impact on ARG transfer, dispersal and evolution.

1.2.4 Using endospore-forming bacteria to investigate ARG

As mentioned above, the use of biological indicators for the reconstruction of past environment and ecosystem history based on sediment records raises the question of their conservation in these records. DNA-based methods might be biased due to the degradation of DNA in sedimentary records (see above). Recently new molecular tools allowing for the study of endospore-forming bacteria and endospores have been developed in our laboratory (see above). Endospores can be considered as time-capsule and thus may give information on past environment. High stability of DNA from endospore-forming bacteria in sediments has been demonstrated. We argue that such a proxy could be used for the detection/quantification of ARG in sediments over time.

Firmicutes are common in human microbiome (Browne et al., 2016; Forster et al., 2019) and encompass a wide variety of pathogens and opportunistic pathogens, especially in the group of *Clostridia* (Aronoff, 2013). Their study has been limited to clinical considerations focusing mostly on (multi-)resistances in pathogens such as *Staphylococcus aureus* (see Lyon & Skurray 1987 for a review; Efuntoye et al. 2011), *Bacillus anthracis* (Cavallo et al., 2002; Jones et al., 2003)*, Clostridium difficile* (see Johanesen et al. 2015 for a review) and other representatives from the genus *Clostrium* (Efuntoye, Bakare & Sowunmi, 2011; Vidor, Awad & Lyras, 2015) or within the class Bacilli (Tarale, Gawande & Jambhulkar, 2015). A recent study also identify an antibiotic resistance pathway specific to the Firmicutes (Coumes-Florens et al., 2011).

However, little is known regarding the prevalence of Firmicutes in the environment, and about the occurrence, frequency and diversity of ARG within Firmicutes. Considering their commonness in human microbiome, their pathogenic potential and their ability to form spores for their preservation and spread, endospore-forming Firmicutes represent interesting candidates for the study of ARG and their prevalence and accumulation in environment. Moreover, the likely underrepresentation of Firmicutes in ecological studies, due to the hard-to-break structures resistant to common extraction method (see above), may suggest they are more abundant and relevant in natural ecosystems than previously thought. Their potential role as reservoir for ARG in environment thus must be evaluated.

In addition to the use of DNA from endospores for studying the accumulation of ARG in sediments over time, recent work conducted in our laboratory highlighted the interest of using endospores for the detection/quantification of ARG in sediments contaminated with trace metal. The impact of the WWTP discharge on the bacterial communities was assessed in the sediments of the Vidy Bay (Lake Geneva), and a potential link with trace metal contamination was explored (Sauvain et al., 2014). Upper layer of sediments near the outlet pipe reflected the influence of the WWTP discharge, showing higher bacterial abundance. Surprisingly results of this study revealed that contaminated sediments were found not only near the WWTP outlet as it had been previously reported, but also relatively far from the outlet pipe. The highest trace metal values were measured in the intermediate area. This spatial distribution is probably due to the heterogeneity of the deposition of heavy metals and organic matter. Regarding the bacterial communities, samples with high trace metal concentrations exhibited a decrease in bacterial abundance and diversity. Endospore-forming bacteria and trace metal concentrations were clearly correlated. In samples with high trace metal concentration *Clostridium* spp. (or close-related OTUs) could represent close to 50% of the total communities. The results of this study suggest a selection mechanism driven by high load of trace metals, leading to the dominance of *Clostridia* and a potential active role of endospores in metals transport.

The most contaminated samples contained mainly endospore-forming Firmicutes that originated from human gut or faeces, and thus were assumed to come from WWTP discharge. In these samples bacterial communities were clearly dominated by Firmicutes, which accounted for up to 62% of the communities. However, Firmicutes abundance did not increase compared to sites near the outlet, only the relative abundance increased. A comparison of the ratio of endospore-forming bacteria in the state of endospores at the different sites revealed an increase of endospores relative abundance in contaminated sediments compared to sediments from the intermediate area and near the outlet pipe, suggesting a selection process that leads to the co-occurrence of trace metals and endospores. The bacterial abundance and the community composition clearly indicated a strong effect of high metal trace contamination on bacteria unable to produce endospores for their self-defense, thus inducing an indirect enrichment in endospore-forming bacteria.

1.2.5 Lake Geneva

Here we propose to focus on Lake Geneva as a model for studying the accumulation of ARG in sediments over time. Lake Geneva is the largest freshwater lake of Western Europe, with a volume of 89 km^3, a surface area of 580 km^2 and a maximum depth of 309 m. The biggest WWTP is located in Lausanne and receives wastewater from both domestic and industrial/clinical sources and discharge water in the Vidy Bay. These releases include treated but also partly untreated sewage. The lake also constitutes the main source of drinking water for the city of Lausanne through the pumping station of St-Sulpice, which is located at less than 4 km of the Vidy Bay (Thevenon & Poté, 2012; Sauvain et al., 2014). Sediments of the Vidy Bay are well described and have demonstrated to be highly impacted by the WWPT, inducing a high degree of organic and inorganic pollution. Contamination to fecal-indicator bacteria, trace metals, nitrogen and phosphorus, antibiotics have been reported (Haller et al., 2011; Czekalski et al., 2012; Thevenon et al., 2012; Czekalski, Gascón Díez & Bürgmann, 2014; Sauvain et al., 2014; Devarajan et al., 2015).

In recent years, two studies initiated by the Eawag (Swiss Federal Institute of Aquatic Science and Technology, Kastanienbaum, Switzerland) investigated the source, occurrence and fate of ARG and MRB in sediments of Lake Geneva, in relation with wastewater treatment. They developed protocols for the quantification of several ARG by qPCR (*sul1, sul2, Sul3, tet(B), tet(M), tet(W)* and *qnrA*). Czekalski et al. (2012) investigated the source and fate of MRB and ARG at different sites before and after their treatment in the WWTP, including wastewater from the main hospital, input and output of the WWTP, as well as sediments and water column near the outlet pipe and a distant site close to a drinking water pump. In addition to culture-dependent methods commonly used for the detection of MRB and ARG, they developed qPCR protocols for the quantification of ARG (*sul1* and *sul2*) directly from DNA extracts. Results indicate that despite wastewater from clinical source is redirected to the WWTP, it appeared that MRB and ARG mostly originate from municipal wastewater, due to the respective volume of wastewater delivered to the WWTP. Unsurprisingly wastewater from hospital contained the highest abundance of MRB and ARG, however their relative abundance was higher in the sediments close to the WWTP outlet, suggesting poor removal and selection process of MRB and ARG during treatment. The comparison between sediment and its overlying water column showed that sediments contained higher abundance of MRB. This points sediments as propitious sites for the accumulation and persistence of ARG.

In the second study, the spatial distribution of ARG was investigated, in relation with the WWTP discharge (Czekalski, Gascón Díez & Bürgmann, 2014). Close to the discharge point, ARG concentration and abundance was up to 200-fold higher than what was measured in the middle of the lake. A correlation between the ARG distribution and the proximity of the outlet pipe has been demonstrated, with an exponential decay of ARG levels (abundance and concentration) with the distance to the outlet. Moreover, accumulation of ARG was not limited to the vicinity of the discharge point but also extended further, in direction of the drinking water pump. These measurements demonstrate the ability of ARG to travel over important distances, what is explained by the directional transport of pollutant in the water column due to the currents. This study also found a high similarity between the bacterial communities from the most ARG-contaminated sites and from untreated wastewater.

Interestingly two studies investigating the dissemination and accumulation of ARG over time were conducted in sediments from the Vidy Bay, based on the analysis of sediment cores. Thevenon et al. (2012) used culture-dependent methods for the detection and quantification of faecal indicator bacteria (FIB), antibiotic resistant bacteria (ARB) and multi-resistant bacteria (MRB). In addition standard PCR were performed for the detection of ARG directly from DNA extracts. They reported an

increase of FIB, MRB and ARB in WWPT-contaminated sediments after 1970, which correlates with the eutrophication of the lake rather than with the implementation of the WWTP. They also detected ARG in most of the sediments tested, even ones distant from the WWTP outlet and older samples. The *aadA* resistance gene was amplified for all the sediment samples, including those not influenced by WWTP effluent water. In contrast, none of the other ARG tested were detectable in the distant core. However, the PCR methods used for the detection of ARG only allow detecting presence/absence of ARG. Although ARG are detectable in sediments even before the 20th century, it is likely that their abundance increased since the implementation of the WWTP. A quantification method would be necessary to discern such trend and rate of accumulation of ARG in sediments over time, and better evaluate the impact of the WWTP.

Devarajan et al. (2015) investigated sediment cores from Lake Geneva and the Vidy Bay in order to evaluate its physico-chemical characteristics (including trace metal concentrations) and quantify ARG and FIB over time. In addition to a general high abundance of ARG in the surface layers, statistical analyses revealed a strong correlation between ARG/FIB abundance and organic matter and some trace metal concentrations (Cu, Pb, Fe, Cd and Hg). In contrast, in their study investigating the spatial distribution of ARG in relationship with the WWTP outlet on the same site, Czekalski et al. (2014) did not establish a clear link (no significant correlation) between Hg levels and ARG abundance, indicating differences in their transport and fate, or additional sources of ARG contamination. Such studies are of prior importance since the natural presence of ARG in environment, the fate of anthropogenic released ARG and their persistence and spread are still widely unknown.

1.3 Objectives of the project

As suggested in the previous lines, this project is divided in two main parts: the first one about paleoecology and the second one about antibiotic resistance genes (ARG). The chapter 2 propose a review of the current knowledge about bacterial spores. A particular emphasis was placed on their environmental significance and their possible application as a paleoecological marker and their use in biotechnology. In chapter 3, we aim first to validate the use of our three-steps extraction protocol (assess the efficiency of the method for the isolation spores) for selectively extracting DNA from spores or other lysis-resistant structures. Bacterial spore community resulting from the application of this tailored method will be compared to the total bacterial community, in order to assess the effect of the method on the bacterial community composition. Secondly, assuming the efficiency of the method for the isolation of spores, we propose to evaluate the diversity, prevalence and geographical distribution pattern of potential spore-formers using a compilation of results from high-throughput sequencing obtained from different studies in three different types of environment. In chapter 4, we aim to validate the use of bacterial DNA as a biomarker for paleoecological studies. Both the DNA from the total and the lysis-resistant community will be evaluated as possible proxy. The consistency of this innovative approach will be assessed by comparison with other geochemical proxies traditionally used in paleoecology. A particular emphasis will be placed on the added value of this DNA-based approach, and on the complementarity of the two communities, with each other and with the geochemical proxies. In chapters 5 and 6, we propose to enlarge the applicability of the molecular methods developed for the study of spores to an actual subject of great concern: the antibiotic resistance genes (ARG). In these chapters, the possible detection of ARG in DNA extracted from spores or lysis-resistant structures, as well as their accumulation over time and their dissemination in the environment through wastewater discharge will be investigated.

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2 Bacterial spores, from ecology to biotechnology

Christophe Paul, Sevasti Filippidou, Isha Jamil, Wafa Kooli, Geoffrey House, Aislinn Estoppey, Mathilda Hayoz, Thomas Junier, Fabio Palmieri, Tina Wunderlin, Anael Lehmann, Saskia Bindschedler, Torsten Vennemann, Patrick S. Chain, Pilar Junier

2019, Advances in Applied Microbiology (Vol. 106, pp. 79–111), Academic Press Inc., DOI: 10.1016/bs.aambs.2018.10.002

Foreword

This chapter is a review summarizing the current knowledge on bacterial spores, with a particular emphasis on their environmental significance and their application in biotechnology. It was published as a book chapter. Spore formation is a common feature widespread among the tree of life. It consists on the ability of an organism to enter a dormant state to withstand unfavorable environmental conditions. As a review, this paper went over the different types of spores known among bacteria, and their cellular process, but also present new discoveries such as an unsuspected diversity of potential spore-formers, and propose different possible applications, from ARG tracking to sustainable agriculture.

Although I am cited as the first author, this publication is a collective effort from the whole laboratory team. The outline of the paper was defined by Professor Pilar Junier, which was also the main contributor in its writing. Each of the co-authors contributed to different extend, by providing specific data and/or drafting paragraphs in relationship with their individual topics. My personal contribution mainly concerns the parts about the investigation of spores (or lysis-resistant structures) in natural environments and their use as a biological marker, including their possible application for paleoecological studies and antibiotic resistance genes tracking. I provided data from different studies and participated to the writing of that part of the paper both as a reviewer and by writing myself some paragraphs.

CHAPTER THREE

Bacterial spores, from ecology to biotechnology

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Contents

Abstract

The production of a highly specialized cell structure called a spore is a remarkable example of a survival strategy displayed by bacteria in response to challenging environmental conditions. The detailed analysis and description of the process of sporulation in selected model organisms have generated a solid background to understand the cellular processes leading to the formation of this specialized cell. However, much less

Advances in Applied Microbiology, Volume 106
ISSN 0065-2164
https://doi.org/10.1016/bs.aambs.2018.10.002

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is known regarding the ecology of spore-formers. This research gap needs to be filled as the feature of resistance has important implications not only on the survival of spore-formers and their ecology, but also on the use of spores for environmental prospection and biotechnological applications.

1. The challenge of bacterial survival

The unpredictability of environmental conditions in natural ecosystems represents a constant challenge to survival. In consequence, it is highly likely that during its lifetime, an individual organism will have to withstand conditions that are suboptimal for growth and reproduction. There are different responses to this phenomenon. The reduced metabolic activity that occurs in dormant cells is one of these, and dormancy therefore can allow the reduction of the fitness cost that environmental stress imposes on a population (Lennon & Jones, 2011). Although dormancy can be beneficial under fluctuating conditions, it can also be a perilous response. It bears a high biological cost, as the individual cell must invest resources in mobilizing the machinery required for the transition from an active to a dormant state (Callahan, Maughan, & Steiner, 2008; van Bodegom, 2007). It is also a risky strategy as the misinterpretation of the physiological and environmental cues that trigger dormancy or allow reactivation to an active state will result in a missed opportunity for growth and reproduction. In spite of these trade-offs, dormant cell forms have been reported in Bacteria, Eukarya, and potentially Archaea (Lennon & Jones, 2011).

Diverse survival strategies in response to challenging environmental conditions have been described for bacterial species. These strategies can constitute a complex coordinated response of the entire community (for example biofilm formation; Kreft, 2004), or favor the survival of individual cells. The latter includes processes involving morphological plasticity (Justice, Hunstad, Cegelski, & Hultgren, 2008) or post-transcriptional modifications that optimize fitness (Zhao et al., 2002). Bacterial dormancy is a well-studied survival strategy that corresponds simultaneously to both categories: community and individual survival. Dormancy can be considered a property of individuals, as not all bacterial species are able to switch from a vegetative to a dormant state. However, dormancy has been described as a community strategy that presupposes an altruistic behavior involving the communication and sacrifice of specific cells before, during and after entering this state (Buerger et al., 2012; Lennon & Jones, 2011; Wolf, Vazirani, & Arkin, 2005).

Dormant microorganisms exhibit a wide range of resting phenotypes, probably reflecting a complex evolutionary history of this trait. A comprehensive definition of dormancy includes any resting period or reversible interruption of the growth or metabolism of an organism. This definition applies both to differentiated dormant cells and to cellular states (for example viable non culturable state) that improve survival (Lennon & Jones, 2011). One of the most common ways of achieving dormancy is through the differentiation of the vegetative cells into resting structures such as spores, conidia, cysts or akinetes. This involves an obvious morphological differentiation involving reduced cell size or a special cell division that marks the formation of the dormant cell (Lennon & Jones, 2011).

Bacterial spores are a highly successful type of dormant cell. Sporulation provides a mechanism by which spore-formers can withstand unfavorable conditions, at temporal and/or spatial scale. Spores are both resistant structures that can persist in a dormant state for extended periods of time, and a vector for increasing dissemination rate, both circumventing local unfavorable conditions that prevent optimal growth (Nicholson, 2002). To date, dormancy in the form of a spore has been thoroughly investigated in four bacterial phyla, and each produces characteristic spore types: Firmicutes (endospores), Actinomycetes (exospores), Cyanobacteria (akinetes), and in the δ-Proteobacteria genus *Myxococcus* (fruiting bodies) (Barton, 2005). Collectively, research in these model groups has generated a strong foundation for exploring the prevalence and implications of this survival strategy (Hutchison, Miller, & Angert, 2014; Lennon & Jones, 2011). Likewise, predicting the ability of an organism to produce spores (or spore-like cells) is often made on the basis of the morphological and genetic information compiled in these well-studied standard model organisms. The process of dormancy in these bacteria has three general stages: initiation, resting, and resuscitation (Lennon & Jones, 2011) (Fig. 1). Initiation is the response to unfavorable changes in the environment. These changes are often detected by a tightly controlled regulatory network because of the high energetic costs of a response to a "false" signal. Therefore, in natural communities a mechanism allowing a responsive switching to initiate dormancy should be favored. The tolerance of the organism to potential stressors in their natural environment would, to a large extent, limit the triggering of responsive switching that in turn would induce dormancy. Likewise, a pathway allowing responsive resuscitation can be predicted. Indeed, dormancy is an adaptive strategy if dormant cells are able to exit it when environmental conditions are favorable and therefore, if efficient mechanisms to trigger exiting dormancy are in place (Lennon & Jones, 2011).

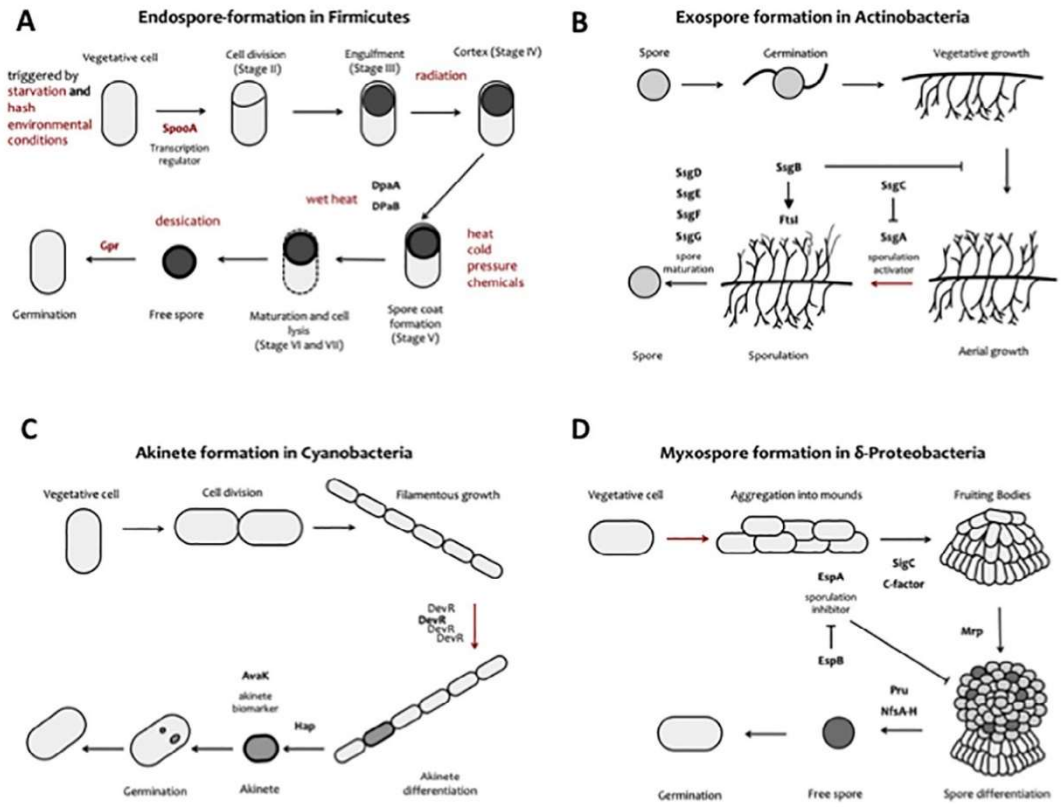

Fig. 1 Schematic representation of the sporulation process in four different bacterial taxa: (A) Firmicutes; (B) Actinobacteria; (C) Cyanobacteria; and (D) *Myxococcus* (mechanism of sporulation due to starvation by the formation of a fruting body). The most relevant genes involved in the respective sporulation pathways are indicated. Bold arrows indicate the key step in which vegetative cells commit to sporulation.

In spite of a large amount of information obtained in model organisms in the laboratory, sporulation in the environment is only recently starting to be investigated in detail. The aim of this review is to provide a comprehensive revision of the information available in the ecology of spore-forming bacteria in natural environments and the potential uses of spore-forming bacteria in biotechnological applications.

2. Mechanisms of bacterial survival through cell differentiation

Among dormant cell forms, the best studied are endospores, which so far have only been found in Firmicutes. Endospore formation is an example of a sophisticated morphophysiological process of cell differentiation. It involves an asymmetric cell division resulting in two cells with distinct morphologies and functions (spore and mother cell), the engulfment of the

pre-spore by the mother cell, and the finally remodeling of this spore within the mother cell (Driks, 2002). Endospores are also an essential cellular state in the case of pathogens or biocontrol agents in which spores are directly involved in human disease (e.g., anthrax, botulism) (Thavaselvam & Vijayaraghavan, 2010) or pathogen control (Tetreau, 2018). In the case of other differentiated dormant cells such as exospores, myxospores or cysts, the mechanisms of formation are much less well understood (Abel-Santos, 2012), limiting our ability to perform comparative studies in order to assess their prevalence. Because of this, endospores are often used as a benchmark for a canonical resistance structure and many resistant cells are compared directly to those. Reports of dormancy involving specialized cellular structures outside these four groups are found in the literature but have often been received with skepticism. Nonetheless, over the last three decades, the formation of cyst-like resting structures has been proposed for diverse non-spore-formers (Sadasivan & Neyra, 1985; Suzina et al., 2004). Examples of resistant cyst-like-producing non-spore-forming bacteria include *Pseudomonas aeruginosa* (Zechman & Casida, 1982), *Micrococcus luteus* (Mulyukin et al., 2009), *Thioalkalivibrio versutus* (Loiko, Soina, Sorokin, Mityushina, & El'-Registan, 2003), *Sinorhizobium meliloti* (Loiko et al., 2011), and *Arthrobacter globiformis* (Mulyukin et al., 2009), among others. Also, recently cyst-like cells have been found in *Serratia ureilytica* Lr5/4, a polyextreme bacterium isolated from a geothermal source in the Atacama Desert (S. Filippidou et al., submitted). However, for most of these cyst-like cells, the process of generation of the resistance structure, the genetic determinants underlying the trait, and the detailed morphology and composition of the resting cell are not yet described.

Based on the reports of resistant cells so far, one could suggest that dormancy in the form of a modified cell is prevalent among diverse bacterial phyla. This has profound evolutionary implications, and it has even been suggested that a common "sporulating" ancestor to bacteria might have existed prior to their evolutionary radiation (Tocheva, Ortega, & Jensen, 2016). Indeed, the ability to enter into a dormant and resistant cellular state would have provided a clear advantage during the evolution of bacteria in the turbulent and highly fluctuating environment early in Earth's history that resulted in exposure to frequent meteoritic bombardments and alternating periods of boiling and freezing temperatures (Nisbet & Sleep, 2001). In order to test this evolutionary scenario, additional models need to be investigated. Therefore, one of the frontiers in research into bacteria that produce dormant cells consists of developing

tailored methods for improving the ability to enrich and culture novel spore-like-forming species (Browne et al., 2016).

The impact of improved methods to assess sporulation in environmental bacteria is remarkable. For example, even in the case of the best-studied group (Firmicutes), the ability to form spores can be discovered in so-called asporogenic (non-spore forming) species. One example of this is the deduction of spore production based on comparative genomic analysis such as in the case of the extremophile *Carboxydothermus hydrogenoformans* followed by the physiological demonstration of spore formation (Wu et al., 2005). Likewise, the recent isolation and characterization of novel species of *Ruminococcus,* previously regarded as a non-spore forming Firmicute genus, have demonstrated their ability to form spores (Mukhopadhya et al., 2018). This illustrates the large scope of work required to address what can be called the "dark *spore-forming* bacterial matter," something particularly relevant in the case of non-Firmicute species. This will constitute a leap forward in our understanding of microbial physiology, and the processes of morphogenesis and cellular differentiation. Novel approaches such as single cell genomics have the potential to allow this (Miller, Weyna, Fong, Lim-Fong, & Kwan, 2016; Rinke et al., 2013) after spores are separated and enriched from the total community. Generating a method to sequence individual spores is still challenging, but should be an approach to be developed in the future.

3. Investigating spores directly from environmental samples

The previous examples considered bacteria forming spores and cyst-like cells that have been cultured and studied in the laboratory. However, it is also possible to investigate the diversity of highly resistant cells directly from environmental samples. Various culture-dependent studies of endospore-forming Firmicutes have shown an interesting distribution pattern suggesting a mismatch between the ecological optima of species and their environmental detection. For example, the revival of endospores from thermophilic endospore-forming Firmicutes species from cold sediments suggests a high dispersal potential associated with the formation of endospores (Bell, Blake, Sherry, Head, & Hubert, 2018; de Rezende et al., 2013; Hubert et al., 2010, 2009; Muller et al., 2014). In this case, dispersal in the form of a dormant endospore reduces the selective pressure

exerted by *in situ* environmental conditions. This is in contrast to the general principle of biogeographical distribution often assigned to microbial communities in which "everything is everywhere but the environment selects," as in the case of metabolically inactive spores, the environmental conditions would not affect community structure (Martiny et al., 2006). This unique biogeographical distribution of endospore-formers as a consequence of their high dispersal potential has been also shown in soils. However, while a study in soil did not show spatial dependence or correlation with *in situ* conditions (Philippot et al., 2009), on the contrary, another one in the soil critical zone has shown a distribution associated to *in situ* environmental parameters (Tsiknia, Paranychianakis, Varouchakis, Moraetis, & Nikolaidis, 2014).

Aside from culture-dependent methods, a suite of novel approaches to study the diversity of endospore-forming Firmicutes in environmental samples have been developed (Bueche et al., 2013; Wunderlin, Junier, Roussel-Delif, Jeanneret, & Junier, 2013). This includes a new approach to carry out targeted DNA metagenomics using sporulation as a functional trait (Wunderlin, Junier, Paul, Jeanneret, & Junier, 2016; Wunderlin, Junier, Roussel-Delif, Jeanneret, & Junier, 2014). A method to enrich lysis-resistant cells was devised based on a combination of physical and chemical treatments inducing the lysis of vegetative cells. Initially developed for the isolation of endospores of Firmicutes (considered to be one of the most lysis-resistant life forms), the method has been applied to various environmental samples with unexpected results. While initial laboratory tests demonstrated a clear enrichment of endospores, results from environmental samples invariably showed extremely diverse communities including many highly abundant taxa that were previously believed to be non-spore formers. Interestingly, these previously unknown spore-formers present in the lysis-resistant fraction of the community were not a main component of the lysis-susceptible community, which indicates they were not simply contaminants due to the incomplete removal of vegetative cells (T. Junier & C. Paul, submitted). Such results suggest a potentially unsuspected diversity of organisms able to form resistant structures. This would give additional support to an evolutionary scenario that includes a widespread prevalence of sporulation (or spore-like formation pathways) in bacterial communities. This is significant considering the potential resilience that these spores and spore-like cells would give bacterial communities in surviving environmental perturbations.

4. The sporobiota: Prevalence of spores in the human microbiota

The concept of sporobiota has recently been used to characterize a particular fraction of the human microbiota that shares the characteristic of producing highly resistant endospores, which facilitates transmission of spore-formers between individuals (Tetz & Tetz, 2017). The need for defining this unique fraction is not only the consequence of the dominance of this group within the human microbiome (Browne et al., 2016), but also the unique emerging features related to the presence of highly resistant spores within this sub-community. These features include resistance to antibiotic treatment by the production of endospores, eliciting detrimental host immune responses, or the possibility of spores acting as an agent of chronic infections, among others (Tetz & Tetz, 2017). A recent human microbiome study based on the enrichment approach mentioned previously (Wunderlin et al., 2016; Wunderlin, Junier, et al., 2014) showed that these lysis-resistant cells are prone to participating in cross-host dissemination and play an important role in the re-colonization after a process affecting the stability of the human habitat (Kearney et al., 2018).

Obesity is the most representative case study in which the presence of potentially endospore-forming bacteria has been linked to health. The presence, and more importantly, a change in the relative frequency of Firmicutes versus Bacteroidetes (increase of the former relative to the latter) has been associated with the microbiota of obese mice (Clarke et al., 2012). Likewise, increases in the frequency of Firmicutes relative to Bacteroidetes have been found to correlate with body mass index in human populations (Clarke et al., 2012; Koliada et al., 2017). Additional studies have been performed in specific cohorts such as pregnant women or children (Santos-Pereira et al., 2018). In the case of children, a general pattern regarding increases in the relative abundance of Firmicutes has not been reported (Santos-Pereira et al., 2018).

5. Potential spore-formers in other microbiomes

The dominance of putative spore-forming groups, especially Firmicutes, appears to be an overarching feature in the microbiomes of mammals (Mao, Zhang, Liu, & Zhu, 2015; Nelson, 2015). A recent study has suggested that captivity affects the mammalian gut microbiome, with

Firmicutes as one of the bacterial phyla changing as a consequence of captivity (McKenzie et al., 2017). These changes can implicate either an increase or a reduction in the abundance of specific groups, and those vary for different host species. In general, captive mammals appear to harbor a higher frequency of anaerobic Bacilli and Clostridia compared to wild counterparts. In addition, other groups potentially comprising spore-forming species such as Actinobacteria and Cyanobacteria appear to also vary in response to captivity, but their prevalence is much lower than that of Firmicutes. Also, a comparison between marine and terrestrial mammals suggests the importance of Firmicutes as a predominant group associated to animals with different dietary preference and habitats. However, an exception to this appears to be a unique signature of reduced abundance of Firmicutes accompanied by increased abundance of Fusobacteria found in carnivorous marine mammals compared to terrestrial mammals (Nelson, Rogers, & Brown, 2013).

Studies considering non-mammalian hosts are currently less common. However, Firmicutes have been reported in specific proportions of the gastrointestinal tract of alligators, but in this case, Fusobacteria appears to be the most relevant phylum and to have taken over some niches usually occupied by Bacteroidetes and Firmicutes in other vertebrates. The relative proportion of Firmicutes varies with feeding-fasting regimes, seasonal feeding, and farm versus wild animals. In the case of farm animals, their feeding regime and deposition of fat appear also to be correlated to changes in the relative abundance of Firmicutes, similar to reports for obesity in other vertebrates (Keenan, Engel, & Elsey, 2013). Firmicutes are also a main component of the gut microbiota of insects, although at a lower degree than in the case of vertebrates (Engel & Moran, 2013; Yun et al., 2014). Evidence for a dynamic change in the prevalence of Firmicutes with developmental stages has also been shown for insects (Chen et al., 2016).

Recent unpublished data from our laboratory suggest that Firmicutes could also be prevalent in the microbiota of fungi. However, determining if bacteria are actually living inside fungal cells instead of closely associated with the exterior of cells can be challenging, and this needs to be investigated further. Nonetheless, by searching for genome-based signals of spore-forming genera within sequenced fungal genomes, we have identified the presence of *Bacillus* spp. in a diverse range of potential fungal hosts (Table 1). This is in agreement with the detection of *Bacillus* and related genera as endobacteria of mycorrhizal fungi (Izumi, Anderson, Alexander, Killham, & Moore, 2006).

Table 1 Signals of spore-forming *Bacillus* species recovered from the genome sequencing data of fungal isolates (291 isolates) sequenced as part of the 1000 Fungal Genomes Project.

Fungal isolate	Fungal phylum (Ascomycota or Basidiomycota) or subphylum	Best match for bacterial signal
Aspergillus sydowii	Ascomycota	*Bacillus* sp. CR71
Cercospora zeae-maydis	Ascomycota	*Bacillus* sp. IHB B 7164
Dissoconium aciculare	Ascomycota	*Bacillus cereus* strain CC-1
Lecythophora sp. AK0013	Ascomycota	*Bacillus coagulans* DSM 1 (ATCC 7050)
Melanomma pulvis-pyrius	Ascomycota	*Bacillus cereus* NC7401
Ophiobolus disseminans	Ascomycota	*Bacillus coagulans* LA204
Penicillium canescens	Ascomycota	*Bacillus velezensis* strain BIM B-439D
Spathaspora passalidarum	Ascomycota	*Bacillus thuringiensis* strain CTC
Pleurotus ostreatus	Basidiomycota	*Bacillus subtilis* strain 50-1
Trichosporon oleaginosus	Basidiomycota	*Bacillus cereus* strain M13 plasmid
Phycomyces blakesleeanus	Mucoromycotina	*Bacillus cereus* ATCC 10987

After quality control and stringent fungal host removal, remaining sequencing reads were assembled into contigs that were then assigned taxonomic classifications using Centrifuge (Kim, Song, Breitwieser, & Salzberg, 2016) and a custom reference database. Contigs that were at least 1000 base pairs long and had at least 80% identity to a *Bacillus* genome in the reference database were considered to have a signal of associated *Bacillus* (11 of 291 isolates). In cases where multiple *Bacillus* species were identified, only the best match is shown based on contig length and the percentage identity match. These results were validated using BLASTn against the NCBI nucleotide database, and the best scoring BLASTn bacterial species or strain is reported here.

6. Prevalence of spore-formers in environmental samples

Despite their potential for global dispersal, the prevalence of many spore-formers in molecular ecology surveys represents a paradox. An example of this current bias is the environmental detection of the phylum Firmicutes. Firmicutes are the second most abundant bacterial phylum according to previous research based on culture collections as well as whole-genome sequencing (Hugenholtz, 2002). Endospore formers are reported to live in

a wide range of environments on Earth's surface and subsurface (Nicholson, 2002; Nicholson, Munakata, Horneck, Melosh, & Setlow, 2000), and the production of endospores should allow these bacteria to be distributed in every habitat on Earth (Martiny et al., 2006). However, an initial phylogenetic assessment of bacterial communities in four metagenomic datasets revealed surprisingly few endospore formers (von Mering et al., 2007). There are at least two potential explanations for this apparently limited abundance. On the one hand, a methodological bias against molecular detection of Firmicutes could explain their under detection (Filippidou et al., 2015). Indeed, it has been shown that a tailored DNA extraction method allows for a better assessment of the presence and diversity of endospore-forming Firmicutes in environmental samples (Wunderlin et al., 2013; Wunderlin, Junier, et al., 2014). On the other hand, the limited detection of endospore-forming Firmicutes in environmental samples might accurately reflect their relative distribution. If this is the case, it is possible that the energetic demands of sporulation (Hofler et al., 2016) and other survival strategies constitute a burden that limits the prevalence of endospore-forming Firmicutes in otherwise non-limiting conditions. Indeed, results obtained from geothermal sources suggest that multiple limiting environmental conditions favor the relative presence of this group (Filippidou et al., 2016). This is consistent with studies showing that bacteria able to withstand harsh environmental conditions have extra genes or even extra chromosomes (Barton, 2005), but this additional genetic material can also be linked to a decrease in fitness under mesophilic conditions (Pope, McHugh, & Gillespie, 2010). For example, larger chromosomes that result in decreased fitness are also observed in endospore-forming bacteria: when there is no environmental pressure for sporulation, Firmicutes tend to lose their extra sporulation-related genes (de Hoon, Eichenberger, & Vitkup, 2010). This would suggest that the fitness cost to preserve the cellular machinery required for increased survival in harsh conditions is likely a better explanation of the distribution patterns of Firmicutes. However, the absence of clear molecular markers to assess the prevalence of other spore-forming clades in environmental molecular datasets limits the generalization of this trend to other groups.

7. Spores and environmental sensing

Dormant bacterial cells have the potential to be highly useful in providing records of past environmental conditions. Paleoecological studies in lake sediments intend to combine physicochemical parameters with biological indicators. Typically, the latter corresponds to organisms producing

identifiable fossilized structures (e.g., pollen grains or siliceous or calcareous microfossils) that must remain unaltered in order to be preserved in the sediments and hence be analyzed at different time-scales (Gorham, Brush, Graumlich, Rosenzweig, & Johnson, 2001). Bacteria are considered as potential proxies of paleoenvironmental conditions given their large cumulative mass, their phylogenetic diversity and the wide range of different metabolisms they represent (Ariztegui, Thomas, & Vuillemin, 2015; Nealson, 1997). Nevertheless, methods for the comprehensive detection of bacterial communities are challenging, especially because of the degradation of DNA with time. Because of this, paleolimnological studies focusing on bacteria have largely been based on fossil pigments (Dressler, Huebener, Goers, Werner, & Selig, 2007; Gorham et al., 2001). An alternative to this is the use of bacterial resting states, a topic reviewed by Renberg and Nilsson (1992). Because spores are able to survive in a dormant state for centuries to thousands of years, these resistant cellular forms are a potential proxy for paleoecological studies.

The isolation and enumeration of viable cells and endospores from sedimentary archives is an old technique (Renberg & Nilsson, 1992). The first published report suggested the presence of viable *Bacillus subtilis* endospores in 320-year-old soil samples (Sneath, 1962). This initial report was then surpassed by later reports suggesting the viability of endospores of *Thermoactinomyces* from archeological excavations containing plant debris deposited between 85 and 125 AD and in 9000-year-old lake sediments (Nilsson & Renberg, 1990; Seaward, Cross, & Unsworth, 1976). A detailed study on the survival and activity of bacteria in a lake sediment core of about 7 m deposited over the past 13,000 years (Lake Constance; Rothfuss, Bender, & Conrad, 1997) shows that below 25 cm all the viable heterotrophic bacteria were present as heat-resistant endospores. Counts of viable spores decreased exponentially with depth and could not be detected below 6 m (about 8900-year-old sediment). Revival of endospores has been used in a series of studies either for paleoecological reconstruction (Bartholomew & Paik, 1966; Renberg & Nilsson, 1992; Rothfuss et al., 1997) or to demonstrate the dispersal of metabolically inactive thermophiles into cold sediments (Bartholomew & Paik, 1966; de Rezende et al., 2013; Hubert et al., 2010). However, culturing studies are biased toward a small cultivable fraction of the community (Amann, Ludwig, & Schleifer, 1995). In a culture-independent approach, the use of an endospore-specific biomarker (dipicolinic acid) suggested that endospores in sediments of the North Sea represent up to 3% of the total prokaryotic community (Fichtel, Koster, Rullkotter, & Sass, 2007). In much older sediment cores, the

abundance of endospores has been estimated to be as high as the total abundance of vegetative cells (Lomstein, Langerhuus, D'Hondt, Jorgensen, & Spivack, 2012). An alternative culture-independent molecular method has been developed, based on the identification of a specific marker for endospore-forming Firmicutes (Wunderlin et al., 2013). This marker, the stage 0 sporulation gene A (*spo0A*), is the master regulator for the sporulation pathway, and has only been reported in Firmicutes (Abecasis et al., 2013; Galperin et al., 2012). This innovative approach was successfully used (in association with other chemical measurements) to reconstruct the history of Lake Geneva, demonstrating the possible use of endospore-forming bacteria as proxies for paleoecological studies. In a first study based on a sedimentary record covering years from 1921 to 2007, changes in the composition of the endospore-forming community reflected the eutrophication process occurring in the lake between 1960 and 1990 (Wunderlin, Corella, et al., 2014). A shift from aerobic groups (*Bacillus*) and facultative anaerobes (*Paenibacillus*) to anaerobic Clostridia (*Clostridium*, *Desulfitobacterium*) was linked to a decrease in oxygen availability, low C/N values and high TOC and Fe/Mn ratio. This trend inverted after 1990, suggesting a recovery of the lake post eutrophication. Interestingly, quantification of *spo0A* gene showed its abundance (copies/gram sediment) was relatively constant with depth compared to the decrease of total DNA and 16S rRNA gene quantification, demonstrating the potential for conservation of DNA from endospore-forming bacteria in sediments. A second study based on the same sedimentary record but with a higher resolution confirmed the effect of eutrophication on community structure and highlighted episodic events of community shift (unpublished results). For example, the increasing use of fossil fuels in the 1950s and 1960s was reflected by an increase in sulfate-reducing bacteria (*Desulfotomaculum* or *Moorella*) (Fig. 2), while punctuated inputs of terrestrial organic matter (1997 and 1924) were indicated by an increase in *Brevibacillus* abundance, together with high TOC values and a C/N ratio in sediments (Fig. 3). Likewise, a cold winter recorded in 1929 was characterized by a high abundance of *Sporomusa*, a genus containing homoacetogenic bacteria whose metabolism is commonly associated with cold anoxic environments.

8. What do spores tell us about antibiotic resistance?

It has been recently suggested that spore-forming bacteria may play an important role in the evolution and spread of antibiotic resistance, due to

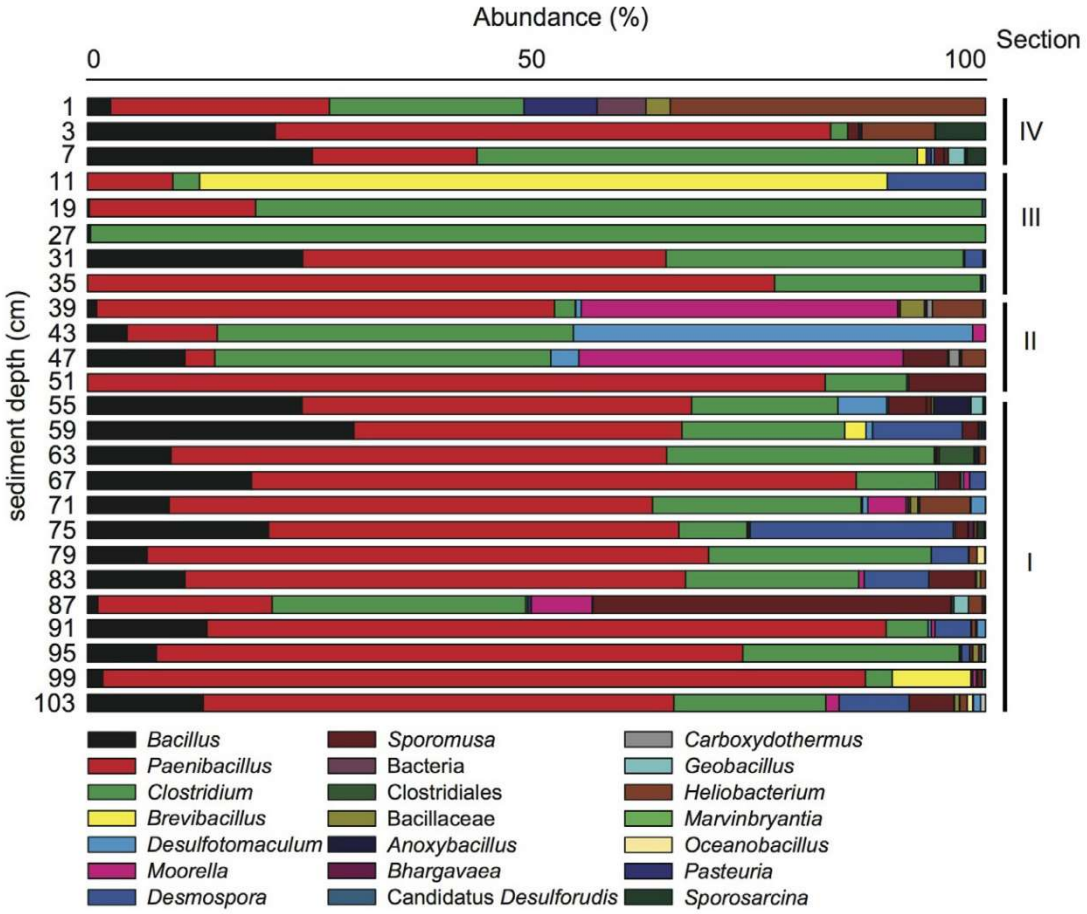

Fig. 2 Temporal changes in the community structure of endospore-forming genera in a sediment core corresponding to the last 100 years of sediment deposition in Lake Geneva. Community structure is based on the analysis of sequences from the stage 0 sporulation gene A (*spo0A*) gene. The *spo0A* sequences were clustered into operational taxonomic units (OTUs) at 97% identity and classified to genus level. The cumulative frequency (in number of sequences) of OTUs belonging to the same genus was added to calculate the percentage values represented for each genus.

their ability to withstand antibiotic treatments and their propensity for dispersal (Bengtsson-Palme, Kristiansson, & Larsson, 2018; Shoemaker & Lennon, 2018; Tetz & Tetz, 2017). Spores and spore-formers are a main component of the human microbiome (Browne et al., 2016) and encompass a wide variety of pathogens and opportunistic pathogens, especially within the group of Clostridia (Aronoff, 2013). Resistance to antibiotics has been reported for clinically relevant spore-formers (Tetz & Tetz, 2017 and references there in) and for several species isolated from the human microbiome (Tetz & Tetz, 2017). In addition, their ability to form a resting state may itself provide resistance to antibiotic treatment (Shoemaker & Lennon, 2018; Tetz & Tetz, 2017). Surviving longer, these organisms have

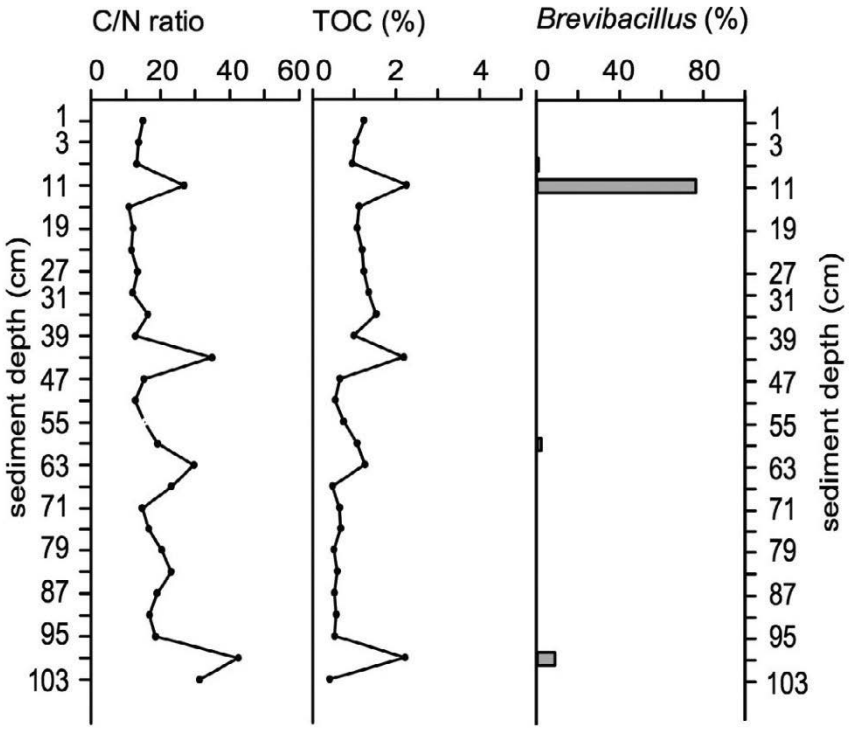

Fig. 3 Depth profile showing the correlation of C/N ratio and total organic carbon (TOC) with the relative abundance of *Brevibacillus*. Samples from a sediment core that has been validate for paleoecological studies obtained in the Rhone Delta from Lake Geneva.

more chances to acquire genetic mutations that confer antibiotic resistance (Levin-Reisman et al., 2017). Moreover, spores not only confer an advantage in the ability to survive stress (such as antibiotic treatment), but they also promote dispersal, thereby overcoming the normal limitations imposed by unfavorable environmental conditions (Bengtsson-Palme et al., 2018; Tetz & Tetz, 2017).

Highly resistant bacteria are assumed to evolve mostly in clinical and animal environments due to the extensive use of antibiotics (Baquero, Martinez, & Canton, 2008). These antibiotic resistant bacteria are notably released in natural ecosystems via sewage effluent, hospital wastewater, agricultural/farming runoff, and/or aquaculture discharge (Marti, Variatza, & Balcazar, 2014; Taylor, Verner-Jeffreys, & Baker-Austin, 2011). Aquatic ecosystems are a key system to investigate the spread of antibiotic resistance genes in human-impacted environments. Due to their uses by humans, aquatic ecosystems constitute: (1) an end-point where resistance genes can accumulate (long-term reservoir), (2) a mix zone where human and environmental organisms can meet and exchange genetic material, and

(3) a pathway for bacterial dissemination and reintroduction into human environment via drinking water supply or recreational activities (Baquero et al., 2008; Taylor et al., 2011). Using DNA extracted from spores as a biological marker, a recent study investigating the dissemination of spore-associated antibiotic resistance genes (ARG) in sediments impacted by the release of treated wastewater demonstrated that spores can be a dispersal vector for the spread of ARG in the environment (Paul et al., 2018). Given the role of spores in cross-individual transmission (Kearney et al., 2018), it appears important to pay attention to this long-lasting fraction of the community.

Although antibiotic resistance is commonly associated with clinical environments, antibiotics and their corresponding resistance mechanisms are ancestral features that evolved long before the therapeutic use of antibiotics by humans (Aminov & Mackie, 2007; Bhullar et al., 2012; D'Costa et al., 2011). Little is known about the natural background of antibiotic resistance in natural ecosystems, and how the release of antibiotics and organisms bearing resistance determinants affects the pool of resistance genes present in human-impacted environments. D'Costa, McGrann, Hughes, and Wright (2006) notably revealed an unsuspected diversity and abundance of ARG in soil microbiomes, highlighting the potential reservoir function of the environment in allowing ARG to spread through various bacterial populations. This is of particular concern since antibiotic resistance can be acquired not only by mutation but also by horizontal gene transfer, which can occur between phylogenetically distant organisms (Courvalin, 1994). The pool of ARG in the environment represents a real threat for human health, with a risk of transmission of ARG from environmental organisms to pathogens and vice versa (Perry, Westman, & Wright, 2014; Taylor et al., 2011). Investigating the environmental accumulation of ARG in sedimentary archives based on DNA-dependent methods is biased, and therefore problematic, because the preservation of DNA in sediments is taxon-dependent and is influenced by multiple biotic and abiotic factors (Boere, Sinninghe Damsté, Rijpstra, Volkman, & Coolen, 2011). Taking advantage of the ability of spores to preserve their genetic material from degradation, a recent study demonstrated a clear correlation between the historical patterns of accumulation of particular ARG within the sporobiota and the timing of medical use of their related antibiotic in lake sediments (Madueno et al., 2018). Likewise, a correlation between these accumulation patterns and specific taxa of the sporobiota was shown in the same study.

9. Biotechnological applications of spore-forming bacteria

9.1 Plant growth promotion

Different groups of beneficial bacteria in the rhizosphere have positive interactions with plants through the colonization of roots and the promotion of plant growth. These bacteria are commonly known as plant growth promoting rhizobacteria (PGPR). PGPR can enhance plant growth either directly or indirectly. Direct mechanisms include fixation of atmospheric nitrogen, solubilization of inorganic phosphorus (Zaidi & Khan, 2007), production of siderophores (Rajkumar, Ae, Prasad, & Freitas, 2010) and of phytohormone (Hayat, Hayat, Irfan, & Ahmad, 2010). Indirect mechanisms include inhibiting the growth of plant pathogens by producing or secreting various chemicals like hydrogen cyanide, phenazines, pyrrol-nitrin and tensin (Bhattacharyya & Jha, 2012), or by promoting beneficial plant-microbe symbioses (e.g., stimulation of mycorrhizae development; Glick, 2012). Among the currently recognized PGPR, *Bacillus* is one of the best-studied examples. There are many species of *Bacillus* that are well known for having plant growth promoting activities (Kumar, Prakash, & Johri, 2011), and these have been commercialized through the availability of *Bacillus*-based biofertilizer products intended to replace chemical fertilizers, for instance, Serenade, Quantum-400, Alinit, Kodiak and Rhizovital (Radhakrishnan, Hashem, & Abd Allah, 2017).

Bacillus species are known to play a vital role in helping plants to withstand the osmotic stress caused by saline water or soil by limiting the uptake of sodium and chloride ions and by enhancing plant growth and seed germination (Jeschke & Wolf, 1988; Qurashi & Sabri, 2013). For instance, *Bacillus licheniformis* A2 has been reported as a salt-tolerant PGPR when applied to peanut plants (Goswami, Dhandhukia, Patel, & Thakker, 2014). Bioinoculation with *Bacillus megaterium* and *Pseudomonas aeruginosa* diminished salt induced cell death in rice (Jha & Subramanian, 2015). Likewise, *Bacillus amyloliquefaciens* NBRISN13, another salt-tolerant PGPR, is reported to mitigate the changes in microbial diversity in the rice rhizosphere in response to salt stress (Nautiyal et al., 2013). *Bacillus* spp. can also help plants by stimulating growth through increasing nutrient acquisition or phytohormone production (Bhattacharyya & Jha, 2012). As an example, screening of 14 different bacterial strains affiliated with *Bacillus* for their plant growth promoting activities showed that 10 out of 14 were capable

Table 2 Screening of 14 endospore forming bacterial strains for plant growth promoting activities.

Bacterial strains	Nitrogen fixation	Proteolysis	Siderophore production	Auxin-like phytohormone production
Bacillus thuringiensis 1312	+	+	+	+
Bacillus thuringiensis 1310	+	+	+	+
Bacillus thuringiensis 1318	+	+	+	–
Bacillus thuringiensis 1070	–	+	+	+
Bacillus thuringiensis 1311	+	+	+	–
Bacillus thuringiensis 1321	+	+	+	–
Bacillus cereus 1055	+	+	+	–
Bacillus cereus 88	–	+	+	–
Bacillus licheniformis	+	+	+	+
Bacillus subtilis	–	+	+	–
Lysinibacillus sphaericus	–	+	–	–
Bacillus polymyxa	+	+	–	–
Bacillus weihenstephanensis	+	+	+	–
Bacillus pumilus	+	+	–	–

+ Stands for activity measured; – stands for lack of the activity.

of nitrogen fixation, whereas all 14 were able to solubilize organic nitrogen (proteolysis), and 11 out of 14 were capable of siderophore production (Table 2). In addition, four of the 14 strains were able to produce auxin-like compounds (Table 2). *Bacillus* spp. are also known to produce cytokinin, gibberellins, and indole acetic acid; all of which can directly or indirectly affect plant growth and yield (Arkhipova, Veselov, Melentiev, Martynenko, & Kudoyarova, 2005; Radhakrishnan & Lee, 2016).

The genus *Bacillus* has also been used as a biocontrol agent against plant pathogens (O'Callaghan, 2016; Widnyana & Javandira, 2016). *Bacillus* spp. can produce a wide range of antiviral, antibacterial and antifungal compounds, which may be important in their interaction with plants and other soil microorganisms. These chemicals have significant commercial potential for increasing agricultural production (Niu et al., 2011). For instance, two antifungal compounds produced by *B. amyloliquefaciens* control the development

of the soil-borne plant pathogen *Fusarium oxysporum*. Moreover, it has been reported in many studies that *Bacillus* spp. are effective biocontrol candidates against diverse fungal diseases that are caused by soil-borne *Rhizoctonia solani*, for instance tomato root rot, damping-off, sheath blight of rice, and potato black scruf (Ben Khedher et al., 2015; Solanki et al., 2012; Yang, Wang, Wang, Chen, & Zhou, 2009). In addition to their use as bio-fungicides, *Bacillus* spp. have also been widely used as bio-insecticides in sustainable agriculture. For instance, *Bacillus thuringenisis* has been used as a broad range bio-insecticide that limits the growth of larvae of pest insects by fatally damaging the midgut epithelium (Radhakrishnan et al., 2017). There are many other *Bacillus* species that have also used in pest management systems, including *B. amyloliquefaciens*, *B. subtilis*, and *B. cereus* (Gadhave & Gange, 2016).

In spite of the potential that plant inoculation with growth-promoting microorganisms has in promoting sustainable agriculture, ensuring the success of inoculations presents many challenges (Ahemad & Kibret, 2014; Bhattacharyya & Jha, 2012; Souza, Ambrosini, & Passaglia, 2015). One of the critical issues involves the survival of bacteria acclimated under laboratory conditions to the harsh conditions in soils. Inoculated microbes have to compete with autochthonous microbial communities (Souza et al., 2015) and are also vulnerable to the relatively low nutrient availability in natural environments compared to laboratory conditions. This often results in a decline in the numbers of the bio-inoculant in the soil overtime (Souza et al., 2015; Trabelsi & Mhamdi, 2013). Moreover, the delivery of these microorganisms in an active form is an additional challenge. Application of carrier materials for the protection of bio inoculants, for instance karnolite, peat, or charcoal, is not only environmentally unfriendly (i.e., extensive mining of peat and related adverse effect on climate) but also costly, making this approach not applicable in agriculture (Arora, Tiwari, & Singh, 2014). These concerns are the incentive to identify more efficient ways of inoculating bacteria into natural environments and increasing their survival. Because of this *Bacillus* and related endospore-forming bacteria may aid development of highly efficient inoculants, as the formation of endospores in response to unfavorable environmental conditions might promote persistence of *Bacillus* in soils (Radhakrishnan et al., 2017).

9.2 Drug delivery

The area of drug delivery is one of the rapidly growing areas of research in biotechnology in which spores are considered as a potential biologically

derived delivery system (Farjadian et al., 2018). Bacterial spores can be useful carries for drugs, but also for nucleic acids or antigens. Some studies have explored the use of bacterial endospores from Firmicutes, as well as vehicles based on proteins from the spore coat for various applications including the presentation of heterologous antigens, vaccination, and delivery of therapeutic agents, among others (Ricca & Cutting, 2003). The direct use of endospores has also been considered, but there is a significant risk associated to germination and unwanted proliferation prior to reaching the target tissue. Nonetheless, endospores from a selected number of *Clostridium* spp. (more specifically *Clostridium histolyticum* and *Clostridium novyi* NT) have been considered in the treatment of cancer, as upon germination and vegetative growth, these species induced lysis of tumor cells (Minton, 2003) or tumor regression (Roberts et al., 2014). Another approach applicable in the case of cancer treatment is the selective expression of specific enzymes upon preferential germination in hypoxic tumors (Heap et al., 2014; Lambin et al., 1998). Recombinant bacterial spores have been also used to generate thermostable vaccines (Duc le, Hong, Fairweather, Ricca, & Cutting, 2003). It appears that a combined expression on the surface of the spore, as well as upon germination, is the best way to stimulate the immune system. Tests conducted so far show that multiple applications are required for immunization (Uyen, Hong, & Cutting, 2007). However, this is an area of research in which progress can be expected as shown by recent publications (Das, Thomas, Garnica, & Dhandayuthapani, 2016; Sibley et al., 2014).

One way in which spores can be used in therapeutic applications is by selecting for spores that respond to specific molecules as inducers of germination. We have tested this approach using oxalate as a signal molecule to trigger germination. Starting from pasteurized soil samples, spores germinating in response to this compound were enriched. The first enrichment clearly showed the presence of oxalate-degrading spore-forming bacteria, which can be purified and characterized (Fig. 4).

9.3 Bioremediation and biomineralization

The term biomineralization refers to the biologically induced and controlled mineralization processes that occur in nature. Microbes are major actors in biomineralization and are responsible for the formation of a wide range of minerals, from iron oxides to carbonates and silicates (Konhauser, Lalonde, & Phoenix, 2008). Although many bacteria have been shown to

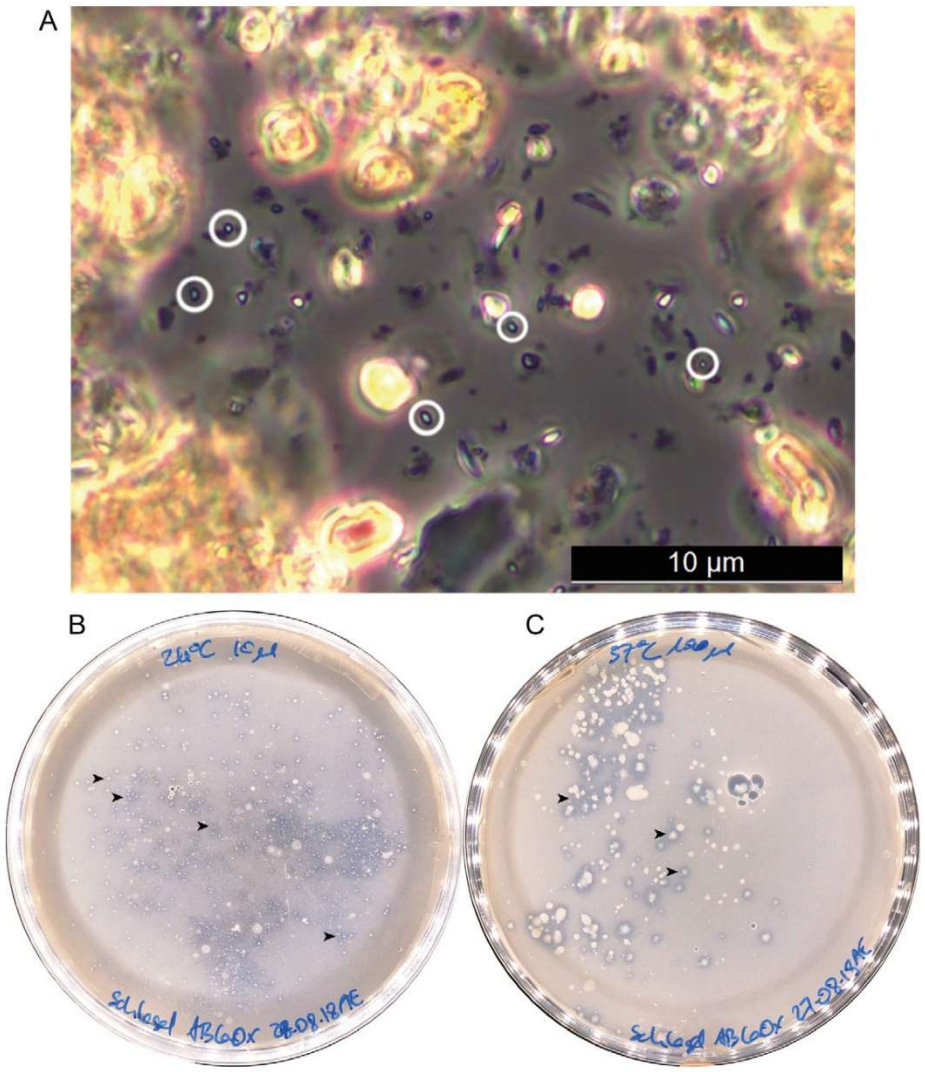

Fig. 4 Isolation of oxalotrophic endospore-forming bacteria from soil by using oxalic acid as a trigger of germination. (A) After a spore separation protocol, the presence of spores was verified by phase contrast microscopy before enrichment in liquid Schlegel AB medium with potassium oxalate as sole carbon and energy source. Spores are shown surrounded by a white circle. (B and C) Initial steps on the isolation of oxalotrophic endospore-forming bacteria on solid Schlegel AB medium with calcium oxalate as sole carbon source. Plates incubated at 24 °C (B) and 37 °C (C). Black arrows indicate the dissolution halos that confirm the presence of oxalotrophic bacteria.

be involved in biomineralization processes, only two cases of spores taking part in redox reactions have been well documented. Spores of *Bacillus* sp. can oxidize manganese (Francis & Tebo, 2002), while the anaerobe *Desulfotomaculum reducens* is able to reduce metals (uranium and iron) in its spore state (Junier et al., 2009). In the former case, this biomineralization process involves the presence of an enzyme responsible for manganese oxidation, multicopper oxidase (Francis & Tebo, 2002), which is localized at

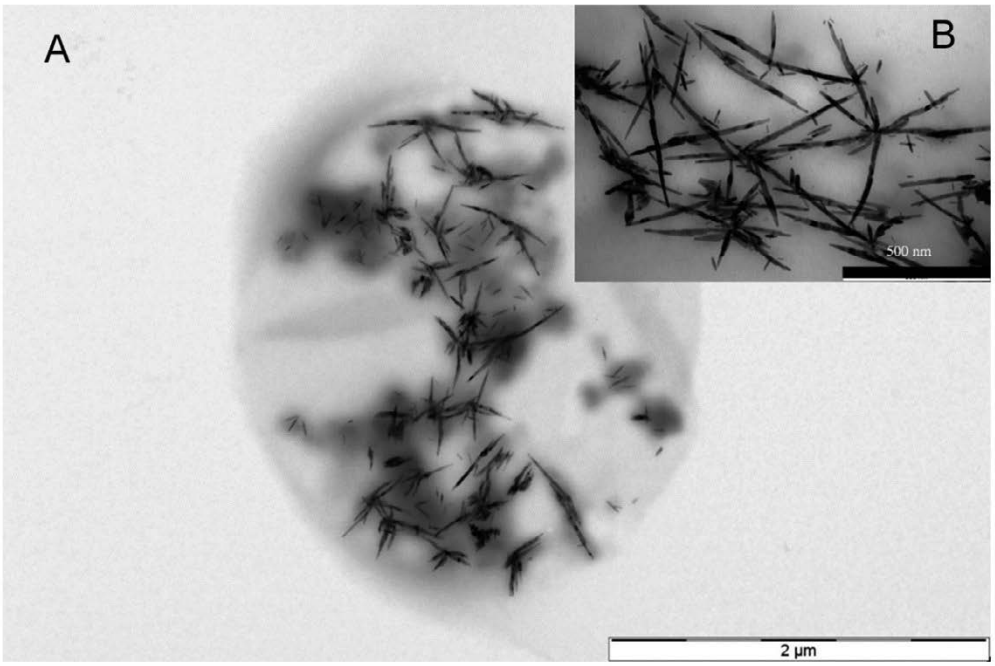

Fig. 5 Transmission electron microscopy (TEM) of Te(0) precipitates produced by *Desulfotomaculum reducens* after reduction of Te(IV). (A) Cell. (B) Close-up to the precipitate.

the exosporium layer of the spore (Francis, Casciotti, & Tebo, 2002). This enzyme was found among numerous *Bacillus* species (Dick, Torpey, Beveridge, & Tebo, 2008), and in agreement, a large fraction of spore-forming species isolated from the environment had shown the ability to oxidize manganese and to tolerate high copper concentrations (Ganesan et al., 2016). In the case of *D. reducens*, in addition of performing a redox active reaction, the spore coat was shown to act as a nucleation center for biomineralization of various metals and radionuclides (Junier et al., 2009) (Fig. 5).

Besides these two examples of reduction and oxidation of metals by spores, the vegetative cells of spore-forming bacteria can also play an important role in metal corrosion and remediation. Indeed, some studies with *Bacillus sphaericus* have shown that the proteinaceous surface layer (S-layer) interacts with uranium and other heavy metals to form nanocomplexes, which could lead to the immobilization and bioremediation of these elements (Merroun et al., 2005).

During a recent study investigating the phenomena of iron corrosion, we isolated spore-forming bacteria from the genus *Bacillus* (Fig. 6). These bacteria were obtained from iron plates that were corroded artificially in a

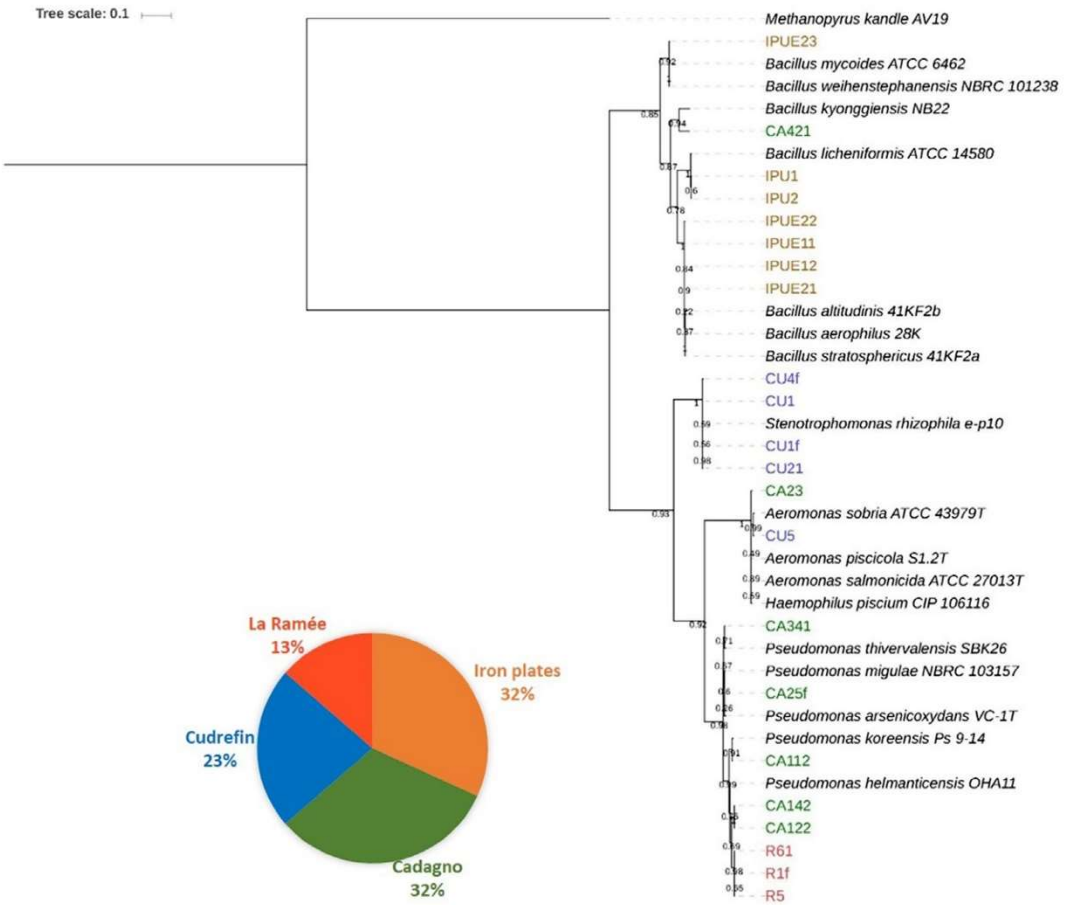

Fig. 6 Maximum likelihood phylogenetic tree describing the phylogenetic relationship between isolates obtained from corroded iron plates. Reference strains are indicated in the tree. The archaeon *Methanopyrus kandleri* was chosen as outgroup. Bootstrap values based on 100 replications were calculated and are expressed on the clades as decimal. The tree shows four main clusters: *Pseudomonas*, *Aeromonas*, *Stenotrophomonas* and *Bacillus*. All the *Bacillus* strains (code IPUE) were obtained from corroded iron plates treated with ethanol and UV. The other clades were enriched from sediments from different origins as described in the sector graph on the left where the percentage of selected isolates from each sampling location is mentioned.

marine environment and immersed in growth medium (LB) for 1 month. Prior to immersion, the plates were washed in a 70% ethanol bath and exposed for 1 h to UV light for cleaning purposes, suggesting their survival in the state of highly resistant spores. The physiological characterization of the strains showed that besides extreme tolerance to salt, most of the strains were able to partially reduce nitrate and iron (Table 3). Iron reduction was couple to the production of ferrous iron minerals, which is an additional example of the potential use of spore-forming bacteria in biomineralization and bioremediation.

Table 3 Summary of the metabolic characteristics of *Bacillus* spp. isolated from corroded iron plates.

Isolate ID	IPU1	IPU2	IPUE11	IPUE12	IPUE21	IPUE22	IPUE23
Nitrate reduction							
Growth NB + KNO_3	+	++	+	+	+	±	+
N_2 production	−	−	−	−	−	−	−
Nitrite production	+	+	−	+	+	+	+
Growth in NaCl (%)							
0	++	++	++	++	++	++	++
2	++	++	+	+	+++	+++	++
4	+	+	+	+	+++	+++	+
6	+	+	+	+	+	+	±
8	+	+	+	+	+	+	−
10	±	±	+	+	+	+	−
Fe(III) reduction with 2% NaCl	−	−	+	−	+	−	+
Optimal temperature (°C)	nd	nd	40	nd	40	nd	30
Optimal pH	nd	nd	6.5–7.5	nd	6.5	nd	7.5

10. Conclusion

Bacterial spores have been known since the dawn of microbiology. Although still seen largely from the perspective of their importance in disease and human health (especially in the case of endospores), bacterial survival in the form of a highly resistance cellular form is likely to be relevant in other ecosystem processes. The basic understanding of the diversity and ecology of spore-formers has direct applications in a diverse range of fields in biotechnology. Nevertheless, in order to advance in this domain, additional tailored tools combining molecular approaches and culture-based techniques are still required. The formation of spores might have been an essential part of the toolkit of early evolving bacteria and is relevant to discuss the resilience of life in a changing planet, as well as the potential existence of life beyond Earth.

Acknowledgments

The authors acknowledge funding from the Swiss National Science Foundation through grants 31003A_179297 (P.J.), CR32I2_162810 (P.J., T.V.), and P2NEP3_178561 (S.F.); the Novartis Foundation through the FreeNovation program; and the U.S. Department of Energy Biological and Environmental Research Division through a Science Focus Area grant to P.S.C. and P.J. (Grant number KP1601010).

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3 Cataloging the diversity of environmental lysis-resistant bacterial cells in environmental samples

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Foreword

Sporulation is a successful survival strategy which allow to withstand unfavorable environmental conditions by entering a dormant state. Little is known about the diversity and environmental significance of spore-formers. This lack of "interest" might possibly be due to methodological limitations and the apparent underestimation of the diversity and ecological significance of this resilient fraction of the community.

In this chapter, we compared results obtained from different studies in three different environments, using the tailored DNA extraction method developed in our laboratory for the study of endospores. This offered new insights on the diversity and distribution of potential spore-formers in the environment.

My personal contribution to this chapter included part of the laboratory work, the analysis of the sequencing data and the bacterial community, the integration and interpretation of the results, and part of the writing.

3.1 Originality-Significance statement

An approach to study lysis-resistant bacteria in a diverse range of environmental samples suggest the existence of resistant cell forms in a diverse set of environmental bacteria for which the production of this type of specialized cell structure is unknown. This might represent a widespread adaptation of phylogenetically diverse bacteria to survive and persist in the environment. If viable, the fraction of lysis-resistant cells of the bacterial community could constitute an essential genetic reservoir from which microbial communities might re-form upon environmental change. Cataloging this fraction of the community is the starting point to device new methods to isolate and characterize the mechanisms by which bacteria achieve long-term survival.

3.2 Summary

In most habitats, fluctuating environmental conditions create periods of compromised survival for metabolically active organisms. In response, various survival strategies have evolved, including the formation of resilient resting cells. In order to catalogue the diversity of this fraction of the total bacterial community, an enrichment approach based on the ability of resting cells to withstand a harsh lysis method prior to DNA extraction was used. Initially, DNA was systematically obtained from sediment samples using the method to enrich lysis-resistant structures and by a method to investigate the total bacterial community. Community composition from each method differed significantly. Species of endospore-forming Firmicutes (for instance *Bacillus*, *Clostridium*, *Paenisporosarcina*) were only detected within the lysis-resistant fraction. This was expected given the tolerance of endospores to lysis. However, and more surprisingly, genera such as *Mesorhizobium* (Proteobacteria) or *Arthrobacter* (Actinobacteria), from which the existence of a resistant cell form is not known, were also highly enriched in the lysis-resistant community. The same method was further applied in a collection of environmental samples from different origins. As it in the case of the comparative dataset, detailed analysis of the detected taxa unearthed many genera hitherto not known to sporulate or to display an equivalent specialized lysis-resistant cell structure. The identification of new taxa capable of generating a lysis-resistant cellular state beyond known spore-formers suggests that production of a durable cell structure might be a widespread adaptation of bacteria to changing environmental conditions.

3.3 Introduction

In various habitats, fluctuating environmental conditions lead to periods during which the survival of physiologically active organisms is compromised. There are different responses to maximize survival under conditions that are suboptimal for growth and reproduction. For instance, dormancy, which is a state of reduced cellular metabolic activity, is one of the strategies reducing the fitness cost of environmental stress on populations (Lennon & Jones 2011). In bacteria, one successful strategy to achieve dormancy is the production of specialized cells such as spores. To date, dormancy in the form of a spore has been thoroughly described in four bacterial phyla, Firmicutes (endospores), Actinomycetes (exospores), Cyanobacteria (akinetes), and in the δ-Proteobacterial order

Myxococcales (myxospores) (Barton 2005). Additionally, obligate intracellular bacteria such as the Chlamydiae (Abdelrahman & Belland 2005) (related to the Planctomycetes) and the Rickettsiae (Rikihisa 2015) (members of the α-Proteobacteria) also produce non-growing differentiated cell types for dispersal. Predicting the ability of an organism to produce spores (or spore-like cells) is often made on the basis of information (both morphological and genetic) reported for these well-studied model organisms (Hutchison et al. 2014; Lennon & Jones 2011).

However, the extent to which model organisms provide an adequate background to the infer the existence of resting cells in environmental bacteria is less clear. This is particularly complicated when considering microbial groups other than endospore-forming Firmicutes, which is the only of the above groups for which a minimal set of the genes required for the formation of a spore has been proposed so far (Abecasis et al. 2013; Galperin et al. 2012). Therefore, investigating sporulation requires a functional proxy for the formation of a durable cell type in the environment. Recently, a method to enrich DNA using sporulation as a functional trait was developed based on the resistance of endospores to vigorous conditions of cellular disruption prior to DNA extraction (Wunderlin et al. 2016; Wunderlin et al. 2014b). The application of this method in sediment samples clearly shows that the differential disruption method results in the enrichment of endospore-forming Firmicutes (Wunderlin et al. 2014b). The same was observed in a recent human microbiome study, which indicated moreover, that lysis-resistant cells are prone to participate in cross-host dissemination and re-colonization after perturbation (Kearney et al. 2018).

In spite of the efficiency of the approach to enrich endospore-forming Firmicutes, surprisingly, endospore formers are not the only group detected by the enrichment method (Wunderlin et al. 2014b). This was initially attributed to potential contamination of the spore fraction with some robust/abundant vegetative cells, and the origin of these unexpected groups was not investigated further. However, the detection of a significant fraction (up to 80% of the sequences) of non-spore forming bacteria in other sediment samples (Madueno et al. 2018) opens up the possibility of a signal for a more diversified community of lysis-resistant bacterial clades within the community. Despite the fact that all DNA extraction methods are intrinsically biased, enriching resistant structures and then characterizing their diversity should provide a more comprehensive insight into community members that are able to generate a lysis-resistant cell form in the environment. Hence, the aim of the present study was to catalogue the diversity of lysis-resistant cells using this specific enrichment method in a diverse set of environmental samples. For this, in order to first eliminate the possibility of contamination of the lysis-resistant fraction with robust/abundant vegetative cells during enrichment, DNA was obtained systematically in both the lysis-resistant and total fraction of the community in a selected set of environmental samples. Afterwards, the enrichment method was applied to environmental samples from very different environments.

3.4 Experimental procedures

3.4.1 Site description and sampling

Samples from Lake Geneva originate from two distinct sampling sites. The first was located in the Rhone Delta, on the eastern side of the lake. A sediment core was retrieved in August 2011 and has been previously dated and described for paleoecological study (core CAN01 (Wunderlin et al. 2014a)).

The second sampling site was the Vidy Bay, located on the shoreline near the city of Lausanne, Switzerland. Samples come from ten sediment cores retrieved between July 2011 and May 2012 and that have been described previously (Bueche 2014; Sauvain et al. 2014).

The Joeri Lakes are located in the Eastern Swiss Alps, in the canton of Graubünden (Switzerland, 46°/46'N and 9°/58'E). Lake I is situated at 2489 m of altitude, has a surface area of 93'700 m^2 and a maximum depth of 10.4 m. Lake XIII is located at an altitude of 2639 m, and has a surface area of 15'400 m^2 and a maximum depth of 10 m (Gabathuler 1999). Samples were collected in August 2016 from sediment cores, surface sediment and soil surrounding the lakes.

Lake Liambezi is located in Namibia at the eastern side of the Caprivi Strip in the complex drainage system of the Kwando and Zambezi rivers. Its southern shore forms the border with Botswana. The Okavango Delta is located in northern Botswana and represents the second largest inland delta in the world, with an area of ~18'000 km^2. Samples from Lake Liambezi, from its affluents/effluents, and from the Okavango Delta, were collected in August 2016 and March 2017, and included sediment cores, surface sediments and river water.

All samples were stored in cold conditions and treated in the shortest possible period before retrieval, including pre-filtering and separation in the field.

3.4.2 DNA extraction

DNA from the lysis-resistant cell fraction was obtained using an indirect three-step extraction method previously described (Wunderlin et al. 2016; Wunderlin et al. 2014b). Briefly, extraction of cells from the sediment was performed by adding 15 mL of Na-Hexa-meta-phosphate to 3 g of wet sediment and homogenizing using Ultra-Thurrax® Tube Drive control (IKA, Stauffen, Germany) for 2x1min at 15'500 rpm. After 10 min of sedimentation, supernatant was retrieved and this first step was repeated with the remaining pellet. Supernatants from the two steps were pooled and centrifuged for 10 min at slow speed (20xg). The supernatant was then filtered onto 0.2 um pore-size nitrocellulose filters (Merck Millipore, Darmstadt, Germany). Water samples were directly filtered onto sterilized filters. Half of the filter was cut (~1.5 g of sediment) to be used for the separation spore-like structures from vegetative cells. Vegetative cells were lysed by a combination of treatments including heat, enzymatic agents (lysozyme) and disrupting chemicals (Tris-EDTA, NaOH, SDS). More specifically, the method consisted of an initial heat treatment at 65 °C for 20 min, followed by a chemical treatment with lysozyme (10 mg/ml) for 60 min and then with a mix of NaOH 0.5 N and SDS 1 % for 60 min. Traces of free DNA from the cells lysed during the enrichment treatment were destroyed by DNase digestion, and lysis-resistance cells were retrieved on a 0.2 um pore-size filter (Merck Millipore, Darmstadt, Germany). DNA extraction was performed using the FastDNA®SPIN kit for soil (MP Biomedicals, USA). A modified protocol including successive bead-beating steps was applied in order to retrieve DNA from the lysis-resistant resting bacterial cells, as shown previously in the case of spores from endospore-forming Firmicutes (Wunderlin et al. 2013). DNA extracts from the different bead beating steps were pooled by ethanol precipitation and resuspended in PCR-grade water. DNA quantification was performed using Qubit® dsDNA HS Assay Kit on a Qubit® 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA).

For the samples from Lake Geneva used for the validation, DNA from the total community was obtained using the FastDNA®SPIN kit for soil (MP Biomedicals, USA), following the same modified DNA extraction protocol and pooling procedure as described above, but without applying the spore-enrichment procedure. DNA quantification was performed using Qubit® dsDNA BR Assay Kit on a Qubit® 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA).

3.4.3 Sequencing and data analysis

16S rRNA gene amplicon sequencing was performed by Fasteris (Geneva, Switzerland), using Illumina MiSeq platform (Illumina, San Diego, USA). Universal primers Bakt_341F (5'-CCTACGGGNGGCWGCAG-3') and Bakt_805R (5'-GACTACHVGGGTATCTAATCC-3') were used for the selection of the hypervariable V3-V4 region (Herlemann et al. 2011). Sequencing data was analyzed using Mothur (Schloss et al. 2009), following the standard procedure of MiSeq SOP (Kozich et al. 2013), with an additional step of singleton removal prior to the clustering in OTUs. The alignment of amplicons and the taxonomic assignment of representative OTUs was performed using the SILVA NR v123 reference database (Quast et al. 2013). A total of 11'387'677 amplicons (2'585'518 unique sequences) were retained after quality filtering and removal of chimeras. At this point, singletons were removed (2'332'236 sequences), as well as unclassifiable sequences or sequences belonging to undesirable lineages (chloroplast, mitochondria, archaea, eukaryote) (99'150 sequences; 2778 unique sequences). The remaining 8'956'291 reads (250'504 unique sequences) were clustered into OTUs, using the OptiClust method (Westcott & Schloss 2017) with an identity threshold of 97 %, leading to the identification of 43'151 OTUs.

The same data treatment for the comparison of lysis-resistant and total community in Lake Geneva gave the following results: 5'026'205 reads after quality filtering and chimera removal (1'016'871 unique sequences), 900'038 singletons, 22'622 (1110 unique sequences) unclassified sequences or from undesirable lineages, and 4'103'545 retained sequences (115'723 unique sequences) clustered in 17'129 OTUs.

The sequences were deposited in GenBank under BioProject accession numbers PRJNA 396429, 396276, and 396277.

3.4.4 Statistical and multivariate analysis

Community and multivariate analyses were performed using R version 3.4.0(Team 2014), and the *phyloseq* and *vegan* packages (McMurdie & Holmes 2013; Oksanen et al. 2017). Principal coordinates analysis (PCoA) were calculated based on Bray-Curtis dissimilarity and Hellinger transformation of the OTU table. Rare OTUs (less than 10 reads) were removed prior to the analysis of the lysis-resistant communities. Rare OTUs were removed as a conservative measure to reduce the background of OTUs representing contamination by abundant members of the non-resistant total community. For the validation experiment (Lake Geneva's samples), the threshold was reduced to 4 reads, as in the case of the validation, evaluating the extent of this potential contamination was important. Difference between the total and lysis-resistant communities was tested using Permutational Multivariate Analysis of Variance (PERMANOVA) using the *Adonis* function from the *vegan* package, based on the same dissimilarity matrix as described above, with 1000 permutations. Distribution pattern of bacterial phyla was analyzed using contour plot of 2D Kernel density estimates, with the *MASS* package (Venables & Ripley 2002). The *Lima* package was used for the display of Venn diagrams (Ritchie et al. 2015).

3.5 Results

3.5.1 Validation of the analysis comparing total and lysis-resistant bacterial communities

In order to first validate if the enrichment method is indeed selecting for lysis-resistant cells and not simply robust or abundant vegetative cells, a set of samples was selected to perform a parallel analysis of the total versus the lysis-resistant community obtained after the enrichment treatment. In the case of contamination, communities from the same sample are expected to be more similar to each other than communities from the same extraction method. Moreover, the most dominant species are expected to be the same between the two extraction methods.

The samples selected for the validation originated from a sediment core that was validated for a paleoecological study in Lake Geneva and that was dated to cover the period between 1921 to 2011 (Wunderlin et al. 2014a). The comparison of the community composition shows a clear separation between the lysis-resistant and total bacterial communities (PCoA Axis 1, explaining 33.2% of the variance; Figure 1). A second axis of separation corresponds to the differentiation of samples before a period in which Lake Geneva became eutrophic (samples dated from before 1955) and thereafter (PCoA Axis 2, explaining 17.1% of the variance; Figure 1A). This was in agreement with a previous study based on a functional gene marker for sporulation (*spo0A*) showing that endospore-forming Firmicutes communities reflected the environmental history and the change in the trophic status of the lake (Wunderlin et al. 2014a). Moreover, PERMANOVA test showed that the total and the lysis-resistant communities were significantly different (p-value<0.001).

To better visualize the groups explaining the separation of the total and lysis-resistant communities, the distribution and density of OTUs (separated by phyla) associated to the separation of the samples were plotted on the PCoA (Figure 1B). This analysis allowed identifying clades for which the density of OTUs is higher in the total community, in the lysis-resistant community, or equally distributed. Acidobacteria is an example of a group containing OTUs only associated to the total community fraction. OTUs belonging to Proteobacteria were distributed in both communities, although represented by individual sets of OTUs for each type of DNA extraction (total versus lysis-resistant). In contrast, OTUs belonging to the Actinobacteria, Chlamydiae, and Firmicutes phyla, were almost exclusively observed in the lysis-resistant fraction. As indicated previously, survival in the form of a highly specialized lysis-resistant spore is well-documented in the literature for species within Actinobacteria and Firmicutes. Chlamydiae are also capable of forming dormant structures called elementary bodies (Abdelrahman & Belland 2005; Hoare et al. 2008), which correspond to a supposed metabolically inert infectious form, fulfilling the definition of a resting cell.

The composition of the communities to the level of genera was analyzed as well. The shift in dominance and community structure of Firmicutes in the lysis-resistant cells community was clearly observed (Supplementary Figure 1). The analysis of the contribution of the 31 most abundant genera in the dataset showed that all known spore-forming bacteria were between five and 25 times more abundant in the lysis-resistant community (Figure 3). The same is the case for three out of 17 the genera that unambiguously correspond to organisms so far not known for the production of spores or other lysis-resistant cellular structures (for instance, *Gaiella*, *Rhizobium* and *Mesorhizobium*). The same was the case for OTUs from seven clades that cannot be unambiguously classified to a specific genus.

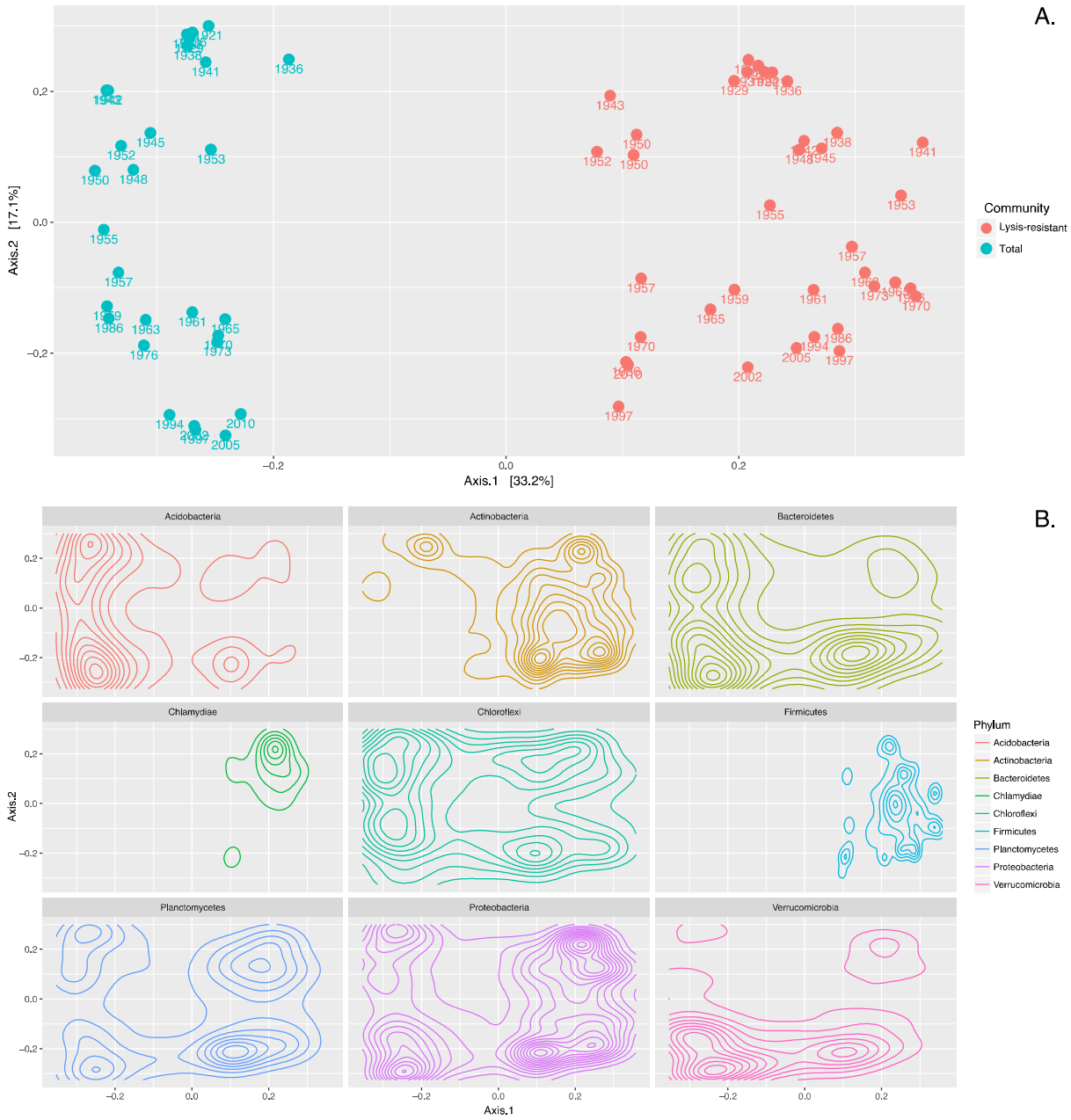

Figure 1: Comparison of the lysis-resistant and total bacterial communities in a sediment core obtained from Lake Geneva, Switzerland. A. Principal coordinate analysis (PCoA) of the communities showing the separation of the lysis-resistant from the total bacterial community. B. Density distribution of OTUs for the 9 most abundant phyla in the dataset projected on the PCoA presented in A (OTUs with minimum 4 reads and phyla > 1% mean relative abundance in the entire dataset).

Among those, OTUs affiliated to the lineages Pir4 (Planctomycetales), D8A-2 (Peptococcaceae), MB-A2-108 (Actinobacteria), and PeM15 (Actinobacteria) were between two and 25 times more abundant in the lysis-resistant community.

Further, the communities of the two most abundant phyla, Proteobacteria and Firmicutes, were compared (Figure 2). In the case of Proteobacteria, OTUs classified as *Anaeromyxobacter* and *Mesorhizobium* are clearly dominant among lysis-resistant structures. In contrast, OTUs classified in these two genera were only rarely found in the total bacterial community. This was particularly remarkable for the most abundant OTU, which was affiliated to *Mesorhizobium* and was more than

1000 times more abundant in the lysis-resistant community (Figure 3), as compared to the total community. In the case of Firmicutes, most of the OTUs were only detected in the lysis-resistant community. The only exception was an OTU affiliated to the genus *Bacillus*, which was more prevalent in the total community. Overall, the analysis strongly suggest that a different fraction of the community is analyzed by applying the enrichment method and that contamination of this community with abundant members of lysis-susceptible total bacterial community is an unlikely explanation for the diversity of previously unknown spore-forming species observed for some of the samples.

3.5.2 Diversity of the environmental bacterial community forming resting cells

To investigate the diversity of environmental bacteria forming lysis-resistant cells, the spore enrichment method was applied to a variety of environmental samples, including sediments, water, and soils. A total of 8'956'291 16S rRNA gene sequences were obtained from treated samples in a diverse set of environments. The sequences were grouped (clustering at 97% identity) into more than 90'000 operational taxonomic units (OTUs). A ranking of the OTUs by sequence counts showed that a large fraction (94%) of the dataset corresponds to OTUs represented by less than 100 reads.

Although a variety of bacterial phyla were represented in the lysis-resistant communities from multiple environments, Actinobacteria, Firmicutes, and Proteobacteria were notably enriched, representing on average 86% of the relative abundance of the bacterial community (Figure 4A). At first sight, this broadly correspond to the phylogenetic diversity of spore forming bacterial phyla. The only exception is the low frequency of Cyanobacteria, which were also detected but not prevalent, which is likely due to the fact that in the environments investigated here photosynthesis is not the most relevant microbial process. Indeed, OTUs affiliated to known spore-forming genera were detected (e.g. *Bacillus, Clostridium, Paenibacillus*, *Lysinibacillus* – Firmicutes-; and *Anaeromyxobacter* – Proteobacteria-(Sanford et al. 2002)), confirming the performance of the enrichment approach to select spore-forming bacteria. However, in many samples, Proteobacteria were largely as abundant (or more so) than the Firmicutes; an unexpected finding since only a limited number of Proteobacterial genera are known to produce spores or similar durable cellular structures (Barton 2005). Closer inspection of the Proteobacteria genera detected provides additional insight into the diversity of groups that resist the cell lysis treatment. Notably, among the 25 most abundant OTUs, several non-spore-forming genera were detected. These included the Proteobacteria *Mesorhizobium, Burkholderia, Thiobacillus* and *Pseudorhodoferax* (Chen et al. 2013); and the Actinobacteria *Arthrobacter* (Ding et al. 2009; Funke et al. 1998) and *Cryobacterium* (Bajerski et al. 2011). These groups represented 42% of the total sequence counts within the 25 most abundant OTUs (Figure 4B). This observation of the enrichment of non- (or rarely-) spore-forming genera even extended to the well-studied Firmicutes (*Turicibacter* (Auchtung et al. 2016; Browne et al. 2016) and *Intestinibacter* (Gerritsen et al. 2014)).

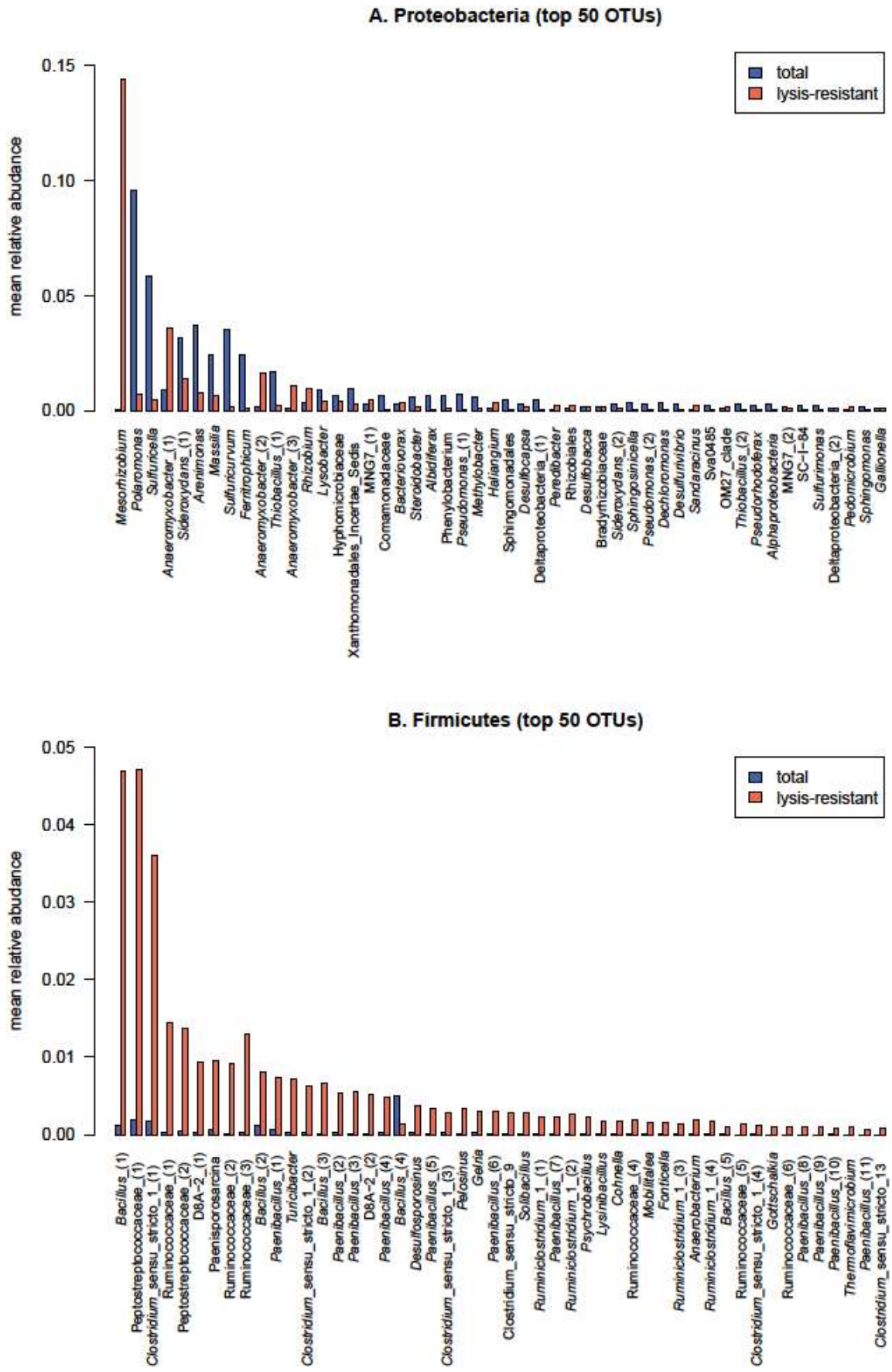

Figure 2: Prevalence of the 50 most abundant OTUs affiliated to the phyla Proteobacteria (A) and Firmicutes (B) comparing the lysis-resistant with the total bacterial communities.

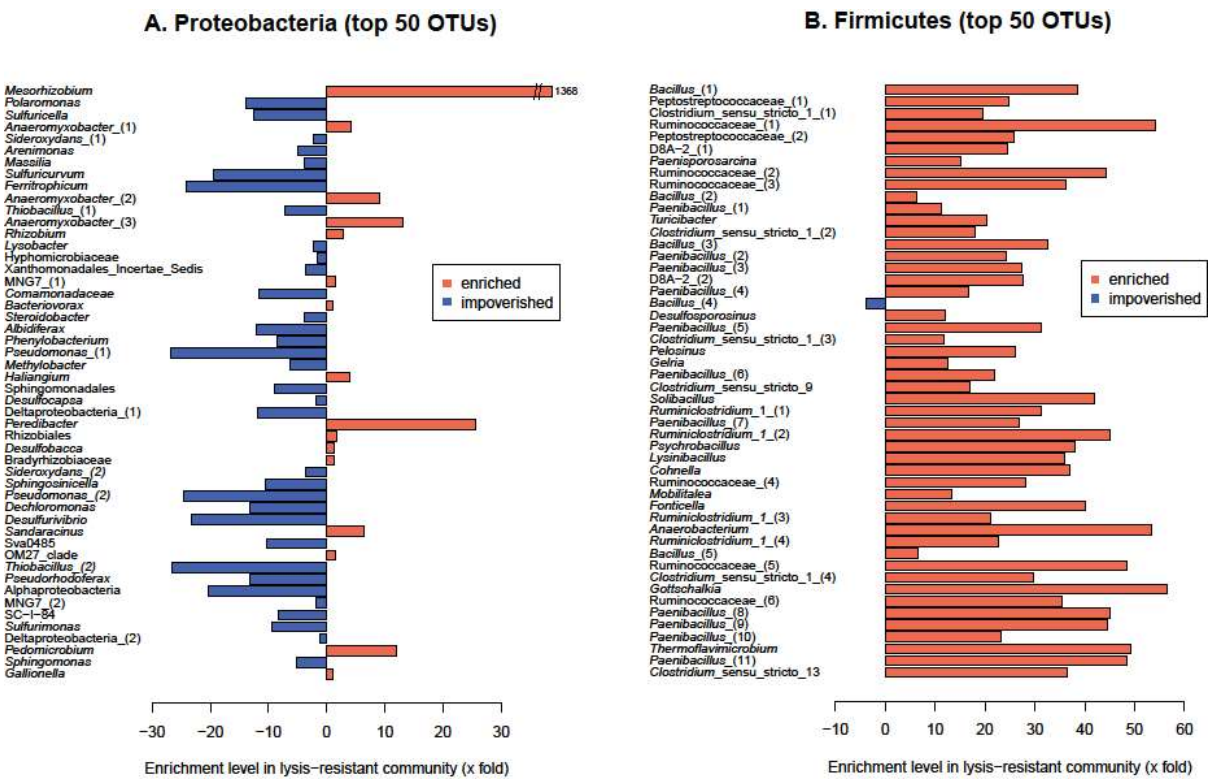

Figure 3: Enrichment level in the lysis-resistant community of the 50 most abundant OTUs affiliated to the phyla Proteobacteria (A) and Firmicutes (B). X axis represents the factor of enrichment (ratio) of each OTUs, with positive and negative values corresponding to respectively an increase and a decrease of the relative abundance in the lysis-resistant community compared to the total community.

3.5.3 Distribution of the bacterial community forming lysis-resistant cells

One of the theoretical hypotheses proposed regarding dormant taxa is their cosmopolitan biogeographical distribution. This derives from the fact that dispersal under a metabolically inactive state should minimize the effect of selective pressure exerted by environmental conditions (Lennon & Jones 2011; Martiny et al. 2006). This hypothesis has been supported by, for example, the presence of thermophilic endospore-forming bacteria in cold environments (de Rezende et al. 2013; Hubert et al. 2009; Muller et al. 2014). Biogeographical distribution of the lysis-resistant community was inferred based on community structure. The sampling included six sites grouped into three types of environments (a tropical river system in Namibia/Botswana; high altitude temperate lakes, and low altitude temperate lakes in Switzerland). These three types of environments were clearly discernible in a principal coordinate analysis (PCoA) based on the structure of the lysis-resistant community (Figure 5A). This geographical signature was still visible for different types of samples (sediment, soil and water). This does not support an overall cosmopolitan distribution of the community forming lysis-resistant cells. Population density is expected to play an important role in the biogeographic distribution pattern of a species, with highly abundant species being more prone to having a cosmopolitan distribution (Lennon & Jones 2011). Therefore, the number of OTUs shared among the three environments (Supplementary Figure 2A) was measured considering the rare and abundant species simultaneously or just the most abundant ones. When rare and abundant OTUs (18515 OTUs) are considered, only 9 % of the OTUs displayed a cosmopolitan distribution. In contrast, when the 1000 most abundant OTUs were analyzed, the percentage of cosmopolitan OTUs increased to 42 %

(Supplementary Figure 2B). This supports the hypothesis of a relationship between species abundance and dispersal potential proposed in the literature (Lennon & Jones 2011).

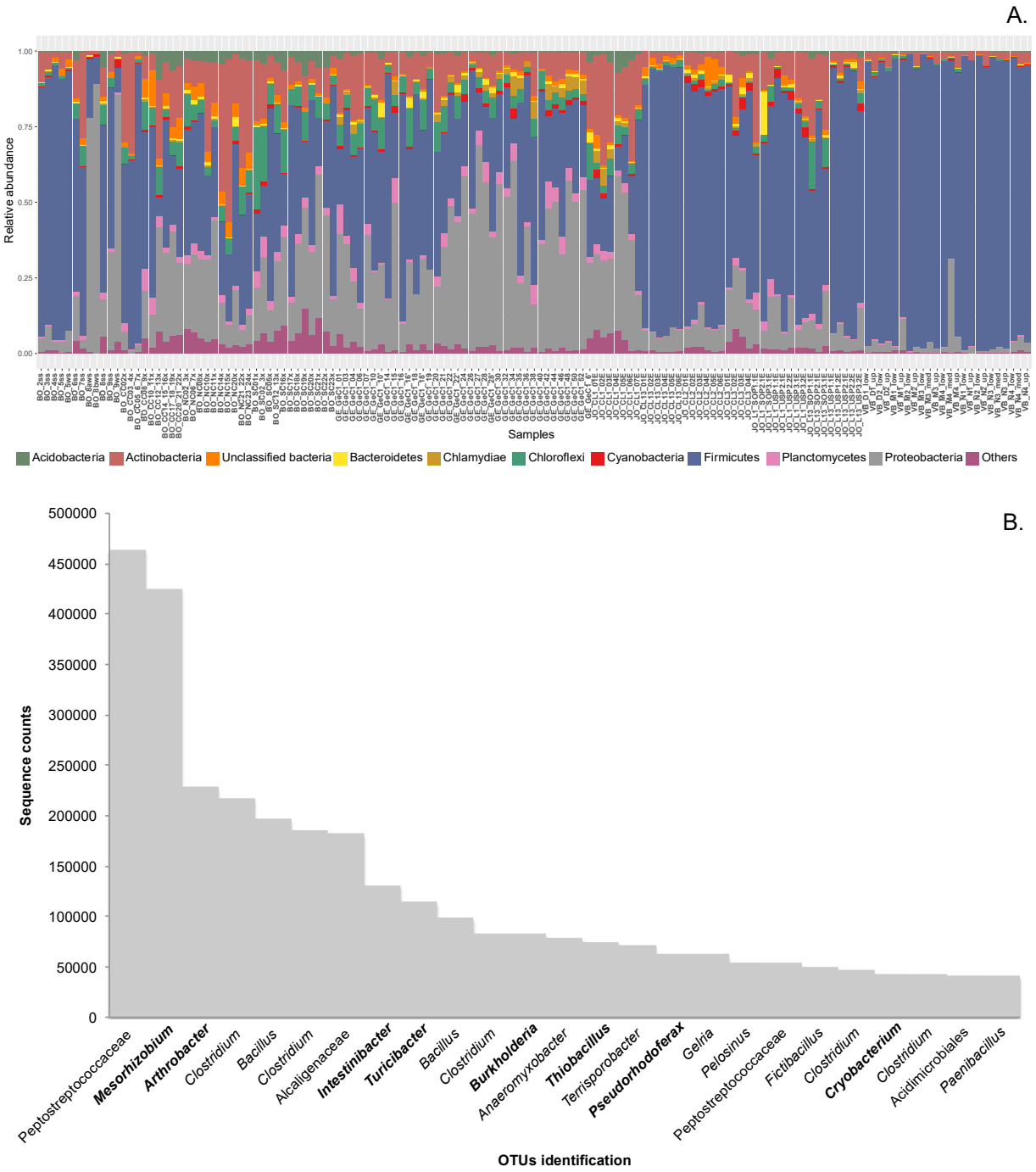

Figure 4: Composition of the environmental lysis-resistant bacterial community in different environments. A. Relative abundance of the most abundant OTUs (over 1% relative abundance) grouped to a phylum level showing the dominance of Firmicutes, Proteobacteria, and Actinobacteria. The relative abundance of these three groups varied greatly between different environmental samples. B. Absolute frequency (in sequence counts) of the 25 most abundant lysis-resistant OTUs. OTU identification is given in the highest taxonomic range (up to genus level) in which the OTUs could be classified. Genera in which spore-formation has not been reported are highlighted in bold.

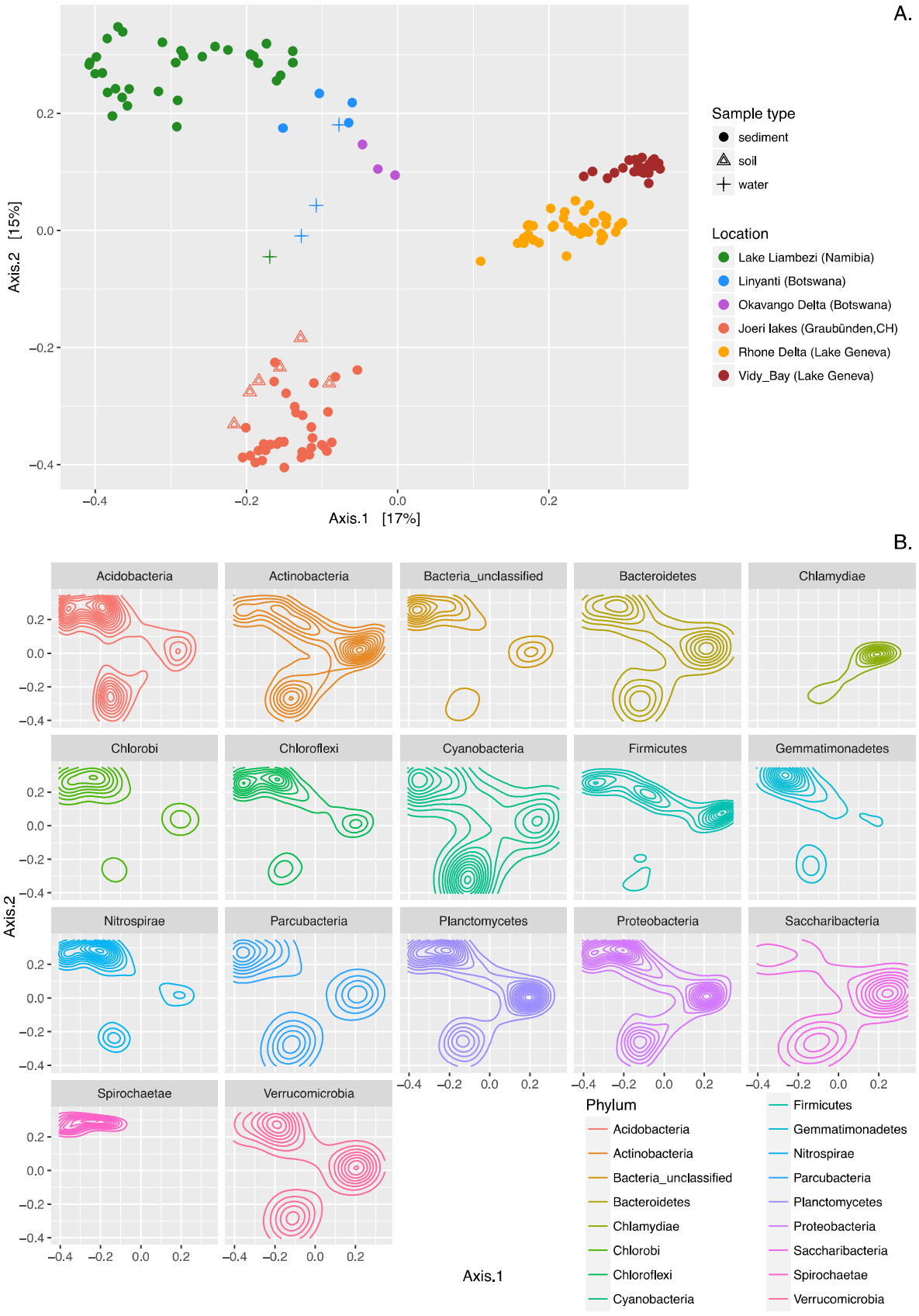

Figure 5: Biogeographical distribution patterns of the lysis-resistant community. A. Principal coordinate analysis showing the similarities of the lysis-resistant communities between different sampling sites. The type of sample (sediment, soil or water sample) is also indicated. B. Gradients indicating the density distribution of OTUs for the most abundant phyla (OTUs with minimum 10 reads and phyla > 0.5% mean relative abundance in the entire dataset) projected on the PCoA presented in A.

The distribution pattern of OTUs affiliated to different bacterial phyla was also analyzed (Figure 5B). Density plots of OTUs belonging to the most abundant phyla (> 0.1% mean relative abundance in the entire dataset) were calculated and displayed in the axis selected in the PCoA of the lysis-resistant communities. OTUs from the phyla Actinobacteria, Cyanobacteria, Planctomycetales, Proteobacteria, and Saccharibacteria appeared in all the environments. For all the other phyla, the density plots suggest a site-specific OUT distribution pattern. In addition, some groups appear to be diagnostic of the environment. For example, the distribution of OTUs affiliated to Chlamydiae, a group that contains strictly obligate intracellular pathogens that require an eukaryotic host to complete their developmental cycle (Abdelrahman & Belland 2005), was denser in samples originating from Lake Geneva. This lake is one of the most human-impacted environments in Western Europe and this group appears to be diagnostic of its anthropogenic impact. The density of OTUs belonging to Spirochaeta, reported as common in natural aquatic environments (Harwood & Canale-Parola 1984), suggest also a site-specific distribution pattern. In this case the highest density of OTUs from this group appears to be a signature of samples from Namibia/Botswana. Literature on Spirochaeta also suggests the existence of ovoid bodies or spore-like spherical bodies, which are resistant and vary in their ability to produce new cells (Thomson & Thomson 1914) or not (Noguchi 1912).

3.6 Discussion

The concept of *sporobiota* has recently proposed to characterize a particular fraction of the human microbiota that shares the characteristic of producing highly resistant endospores, which facilitate transmission between individuals (Tetz & Tetz 2017). The need for defining this fraction of the microbial community is the result not only of the dominance of endospore-forming Firmicutes within the human microbiome (Browne et al. 2016), but also of the unique ecological features emerging in response to the presence of highly resistant endospores. For instance, inherent resistance to antibiotics, detrimental host immune responses, or the possibility of spores acting as an agent of chronic infections, are all ecological properties associated to the production of endospores (Tetz & Tetz 2017). The original definition restricts the term to Firmicutes, the only phylum known to produce heat-resistant endospores. However, and despite the dominance of Firmicutes in the dataset produced here, the results suggest that in the case of environmental samples the concept could be expanded to other types of resting, or spore-like, resistant structures. The results from diverse environmental samples show a diverse community that includes a large number of clades for which experimental evidence for spore (or another lysis-resistant cell form) is still missing.

Detecting spores or spore-like cells would be the incontrovertible evidence of these non-spore-formers to produce a durable lysis-resistant specialized cellular structure. However, in order to accomplish this, enrichment and culturing of these organisms and a better genetic characterization of non-standard spore-forming model organisms are still required. Progress in this area is slow and might suffer from many caveats. For instance, a controversial case involving the proposition of sporulation in a novel clade corresponded to the observation of phase-bright bodies in mycobacteria (Ghosh et al. 2009), which was later dismissed as an artefact on the basis of the lack of replicability and the absence of the minimal set of sporulation genes in mycobacteria. The origin of the initial observation was suggested to be contamination by an endospore-forming strain in the original mycobacterial culture (Traag et al. 2010). Likewise, one of our own isolates, which was initially identified as *Arthrobacter* (strain 13agg1) and in which phase-bright bodies were observed by microscopy, was later shown to have been replaced by a *Bacillus* spp., showing the risks of undoubtedly demonstrating sporulation in environmental strains. Therefore, one of the next frontiers in research into this fraction of the

microbial community consists in developing tailored methods for improving not only molecular assessment (Filippidou et al. 2015; Wunderlin et al. 2016), but also our ability to enrich and culture novel spore-like-forming species (Browne et al. 2016). For example, very recently, isolation and characterization of novel species of *Ruminococcus,* previously regarded as a non-spore forming Firmicute genus, has shown their ability to form spores (Mukhopadhya et al. 2018). Moreover, the formation of resting cells by a large diversity of bacteria has been suggested as the consequence of long-term anabiosis (Suzina et al. 2004; Suzina et al. 2006), but has not been studied further.

Nonetheless, the results from the environmental samples suggest a potentially large diversity of bacteria producing lysis-resistant structures, which need to be explored in more detail. Commensurate with the finding that most bacterial taxa are not culturable, most sporulating taxa likely only produce spores in their natural environment. Therefore, expanding the catalogue of lysis-resistant species to other phylogenetic clades allows further investigation of the ecological properties of this unique fraction of the bacterial community. This includes its role in environmental connectivity, microbial biogeography, diversity maintenance and microbial evolution (Lennon & Jones 2011; Shoemaker & Lennon 2018). For example, revival of thermophilic endospore-forming bacteria has been used to investigate the effect of oceanic currents on the dispersal of bacteria in marine environments (de Rezende et al. 2013; Hubert et al. 2009; Muller et al. 2014). Likewise, a correlation between the historical patterns of accumulation of specific antibiotic resistance genes and the lysis-resistant communities in lake sediments have been demonstrated (Madueno et al. 2018). The latter illustrates the importance of this fraction of the microbial community in the investigation of the response of biological communities to changing environmental conditions, in this case the effect of antibiotics as a selective pressure during the so-called antibiotic age (Madueno et al. 2018). This has important implications regarding the evolution of infectious diseases (Shoemaker & Lennon 2018), in which spores could constitute a long-term reservoir of pathogenic organisms or of resistance genes (Tetz & Tetz 2017).

3.7 Conclusion

Survival is a universal biological theme. Cataloging the diversity of structures and mechanisms allowing bacterial survival under adverse conditions is essential, as well as is the understanding of the environmental cues involved in both dormancy and revival. This can be investigated further by comparing spore-like forming species and geochemical parameters used to qualitatively evaluate environmental change. We inhabit a planet that is expected to undergo increasing episodes of punctuated environmental stress. Under these conditions, sporulation (or the production of other resistant cell forms) might be an adaptive trait that will be under a strong positive selection with unknown consequences in the dynamics of microbial populations in the environment (Shoemaker & Lennon 2018). Dormant populations might constitute a seed bank from which new communities emerge after perturbation. Microorganisms are at the base of the functioning of the biosphere, therefore, the unique community of bacteria forming resting cells cannot be ignored if we want to improve our ability to predict the response of the biosphere to environmental change.

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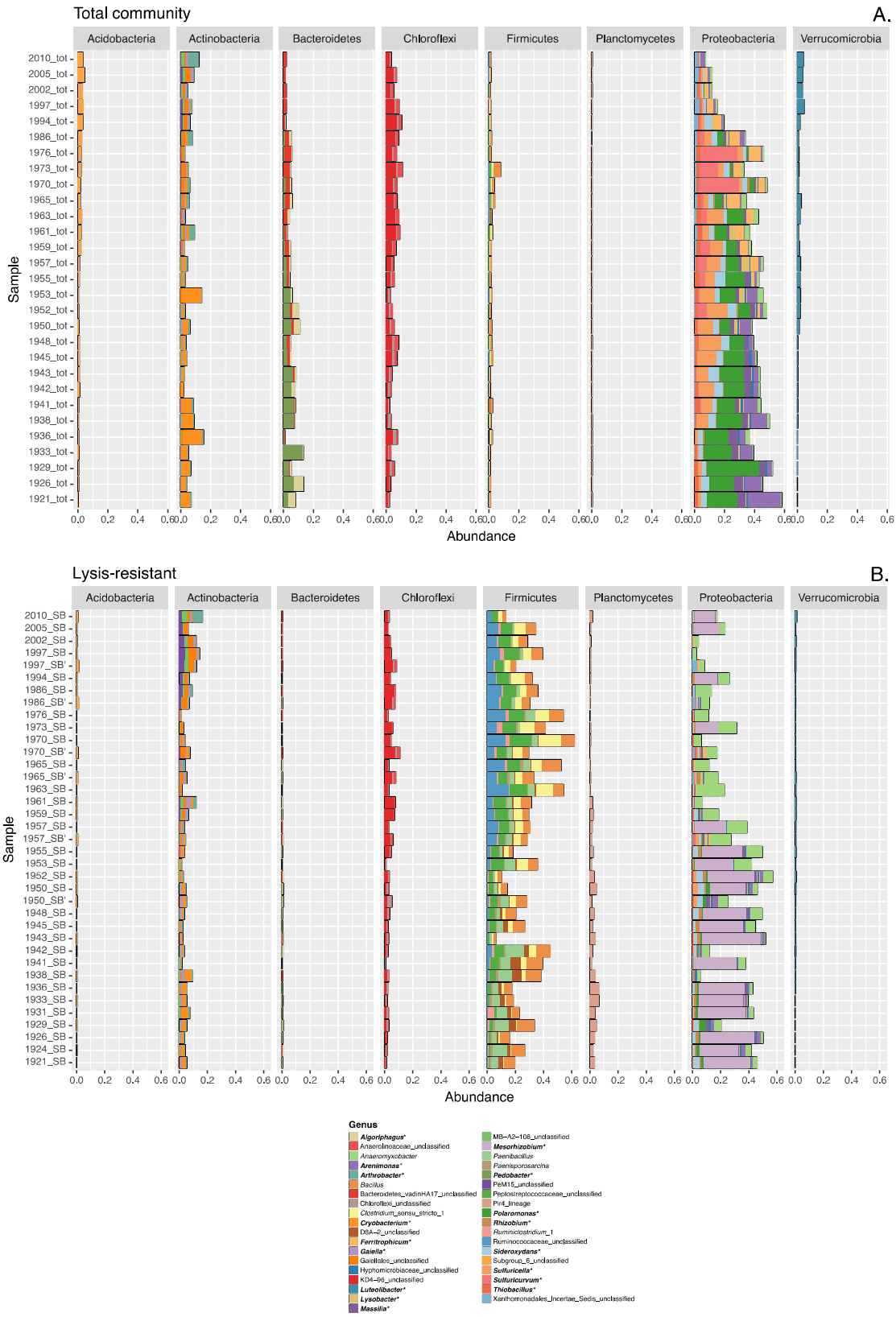
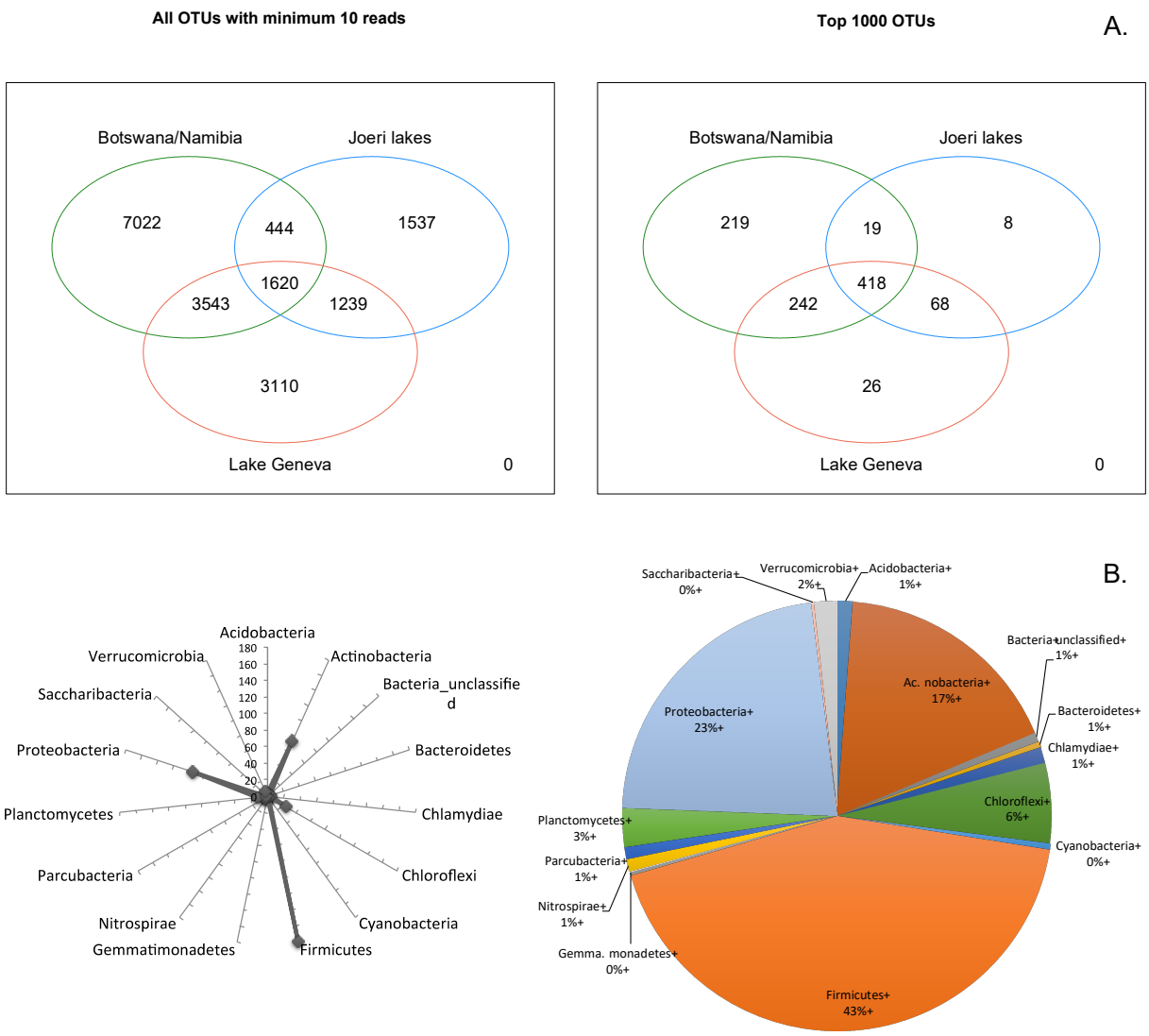
3.9 Supplementary material



Supplementary Figure 1: Comparison of the total and lysis-resistant communities in a sediment core obtained from the Rhone Delta in Lake Geneva, Switzerland. For this analysis, phyla representing at least 1% of the community were selected. From this subset, the 37 most abundant genera composing the total (A) and lysis-resistant (B) communities are indicated.



Supplementary Figure 2: Cosmopolitan distribution of the lysis-resistant community. A. Venn diagrams showing the number of shared OTUs between the three sampling locations. On the right all OTUs with an abundance of minimal of 10 sequence reads were considered (18515 OTUs). On the left only the 1000 most abundant OTUs were considered. B. Phyla composition the cosmopolitan lysis-resistant community considering the 1000 most abundant OTUs. The relative abundance of each Phylum is displayed on the right.

4 Cross correlation of bacterial communities and geological proxies in paleoecology: a holistic approach for the study of past environmental history

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* These authors contributed equally to this work

Foreword

To date, no general marker, representative for all bacteria, exists in paleoecology. This is a main gap since bacteria are the most abundant and diverse group of organisms among all domains of life. They colonized all ecosystems, even the most extremes, and are involved in all biochemical cycling of elements. Their community are shaped by the environment, which is shaped by bacterial activity in response. A biological marker targeting bacteria would be undoubtedly of main interest for paleoecological studies. In the past decades, due to advances in the field of metagenomics, the use of DNA have been proposed as a possible bio-indicator. However, DNA and vegetative cells are subjected to degradation, and therefore, their use remains uncertain. Due to their ability to withstand degradation for extended times, spores or other lysis-resistant structures might represent an ideal marker for paleoecology.

In this chapter, we evaluated the possible use of bacterial DNA as a marker for paleoecological studies. Both DNA extracted from the total and the lysis-resistant community has been investigated, in complement to other geochemical proxies commonly used.

This work is the result of a close collaboration with Anaël Lehmann, a PhD student from the Institute of Earth Surface Dynamics, in the University of Lausanne. Although we both contributed to most of the laboratory work and the analysis of the results, each one was responsible for his domain of expertise. Therefore, my personal contribution mainly consisted in the analysis of the sequencing data and the bacterial community. The integration and interpretation of the results as well as the writing of the manuscript were done jointly.

4.1 Abstract

The study of the impact of past environmental conditions on ecosystem evolution provides valuable information to better predict the impact of environmental change and the response of ecosystems. Bacteria are key organisms for ecosystem functioning, and their community composition is both a reflect and a changing driver of environmental parameters. Thus, investigating changes in the bacterial community might undoubtedly increase our ability to reconstruct ecosystem history and predict the impact of environmental change. In this study, we demonstrate the use of bacterial DNA, extracted from both the total and the lysis-resistant fraction of the community, as a complementary proxy for reconstructing the history of the ephemeral Lake Liambezi (Namibia). A combination of geochemical and sedimentological proxies was coupled to the analysis of the bacterial community. By allowing to establish a common timeline, the analysis of the bacterial community helped to recalibrate the dating of three sediment cores and to refine the age model for the entire ecosystem. The reconstructed climate evolution over the past 5500 years highlighted changes in the hydrological lake regime (from a fen to a lake) resulting from the alternation of dry and wet climatic periods. Changes in both the total and the lysis-resistant community reflected changes in the environmental conditions, providing complementarity information. The high abundance of sulfur-oxidizing and thermophilic bacteria revealed an active sulfur cycling and likely hydrothermal activity within the lake. The latter is supported by barium measurements. This study applying this multidisciplinary approach demonstrates the potential of bacterial DNA in paleoecology.

4.2 Introduction

Paleoecological studies using sedimentary records of aquatic ecosystems provide a unique temporal perspective on patterns, causes, and rates of ecological change due to natural hydrologic and climatic variability, and anthropogenic activity over various time scales. Paleoecology is not only relevant to investigate the past history of an ecosystem and its response to change, but also to determine baseline conditions used as targets of restoration policies (Willard & Cronin, 2007). Various biological, chemical and physical proxies are traditionally employed for investigating ecosystem history (Gorham et al., 2001). However, the level of complementarity of different proxies is still not clearly determined.

The use of bacteria for paleoecological studies is becoming of particular interest thanks to technological advances that allow more than ever the rapid assessment of bacterial diversity in environmental samples (Nealson, 1997). Bacteria are the most abundant organisms on earth and have, amongst other environments, also a considerable cumulative mass in lakes (water column and sediment) (Mrozik, Nowak & Piotrowska-Seget, 2014). Bacterial communities in lakes are phylogenetically diverse and thanks to their functional plasticity, colonizing every available habitat (Nealson, 1997). Because of the role of bacteria on biogeochemical cycling of elements (Madsen, 2011), their interaction with the environment is complex, by simultaneously changing the environment and responding to environmental changes (Chen et al. 2015 and references therein). Therefore, analyzing how the structure of bacterial communities change over time might provide valuable information about the evolution of environmental conditions, the resilience of an ecosystem, or the impact of anthropogenic activity.

To date, most studies using proxies related to bacteria have focused on fossil photosynthetic pigments (Gorham et al., 2001; Dreßler et al., 2007). However, photosynthetic pigments encompass only phototrophic populations and represent but a small fraction of the bacterial community. Technological

advances in the field of environmental genomics now allow for studies of microbial communities and evaluation of microbial diversity from sedimentary records. To achieve this, environmental genomics uses “ancient” or “fossil” DNA as a proxy (Coolen & Gibson, 2009; Boere et al., 2011; Fernandez-Carazo et al., 2013; Pansu et al., 2015). However, the use of DNA-dependent methods for the study of bacterial communities in paleoecology raises the question of DNA preservation in sediments. DNA degradation has been shown to be influenced by multiple biotic and abiotic factors, leading to a differential degradation rate across taxa (Boere et al. 2011 and references therein). Moreover, *in-situ* activity (Nealson, 1997; Haglund et al., 2003) and possible modifications of the community structure during sediment diagenesis could lead to a misinterpretation of the environmental conditions at the time of deposition. Alternatively, extracting DNA from bacterial resting states, and notably endospores and other spore-like structures, might avoid these limitations (Wunderlin et al., 2014a). The use of such structures has been examined and proposed as an alternative to total DNA-based analyses (Renberg & Nilsson, 1992; Wunderlin et al., 2014a; Paul et al., 2019). For instances, endospores are highly specialized cellular structures that can resist desiccation, are robust to natural temperature variations, and radiation (Nicholson et al. 2000 and references therein). Endospores have been reactivated from sediments as old as 9’000 years (Nilsson & Renberg, 1990; Rothfuss, Bender & Conrad, 1997). More “controversial” studies have reported the revival of *Bacillus* endospores from 25- to 40-million-year-old (Cano & Borucki, 1995) and 250 million-year-old records (Vreeland, Rosenzweig & Powers, 2000). Moreover, previous studies have shown the use of endospores as good biological indicators. For instance, *Clostridum perfringens* or *Thermoactinomyces vulgaris* have been proposed as indicators for sewage pollution or agricultural activity, respectively (Renberg & Nilsson 1992 and references therein). However, all of the above studies have been based on the revival and culturing of endospores, which might be biased, due to the small fraction of organisms amenable to cultivation (Amann, Ludwig & Schleifer, 1995).

The development of molecular tools to specifically enrich in lysis-resistant cells created new venues in this field of research. These methods include the development of protocol adapted to obtain DNA from highly resistant structures (Wunderlin et al., 2013) and a treatment for the physical separation of endospores from vegetative cells prior to DNA extraction (Wunderlin et al., 2014b). A combination of these methods was successfully used to assess the impact of eutrophication (Wunderlin et al., 2014a) and the antibiotic era on the bacterial communities in sediments of Lake Geneva (Madueño et al., 2018). The aim of this study is to broaden the application of this approach and test its complementarity to more traditional paleoecological proxies in another aquatic system. The site selected is the Lake Liambezi, located in Namibia at the border with Botswana. Lake Liambezi is an ephemeral lake that floods seasonally, depending on the annual precipitation pattern of the region. The lake is a major resource of freshwater for the surrounding villages and communities of farmers and fishermen (Tweddle et al., 2011; Mutelo, Murwira & Kileshye-Onema, 2013; Peel et al., 2015). To date, except for a brief description made by Seaman et al. (1978), the lake has been poorly studied. No study has yet investigated the type of sediment or attempted the dating of the ecosystem by assessing sedimentation rate or dating. Furthermore, the reconstruction of the climatic history for the last 5500 years for the region varies slightly from one site to another (Burrough et al., 2007). Because Lake Liambezi is essential to the surrounding populations and its surface (or also groundwater resources) varies greatly (going as far as to entirely dry up), a study of its recent history may help to better understand its global dynamics and its evolution in relation to climate.

In the present study, the analysis of bacterial communities, both the total and the lysis-resistant fraction, was assessed as a general bacterial biomarker for reconstructing the ecosystem history and climatic change of Lake Liambezi. Moreover, a traditional approach including standard geochemical,

sedimentological and isotopic analyses has been applied in order to determine the complementarity of this biological markers for paleoecological reconstruction.

4.3 Material and methods

4.3.1 Regional settings and lake description

Lake Liambezi is located in Namibia, at the eastern side of the Caprivi Strip (Figure 1A). The lake is part of a complex drainage system that includes the Kwando and Zambezi rivers. Its Southern shore makes up the border between Namibia and Botswana. It receives water from the Chobe River (a tribute of Zambezi River), Bukalo Channel (another tribute of Zambezi River), and Linyanti River (or Swamps, natural deviation of Kwando River), as well as rainwater and local runoff water (Figure 1B).

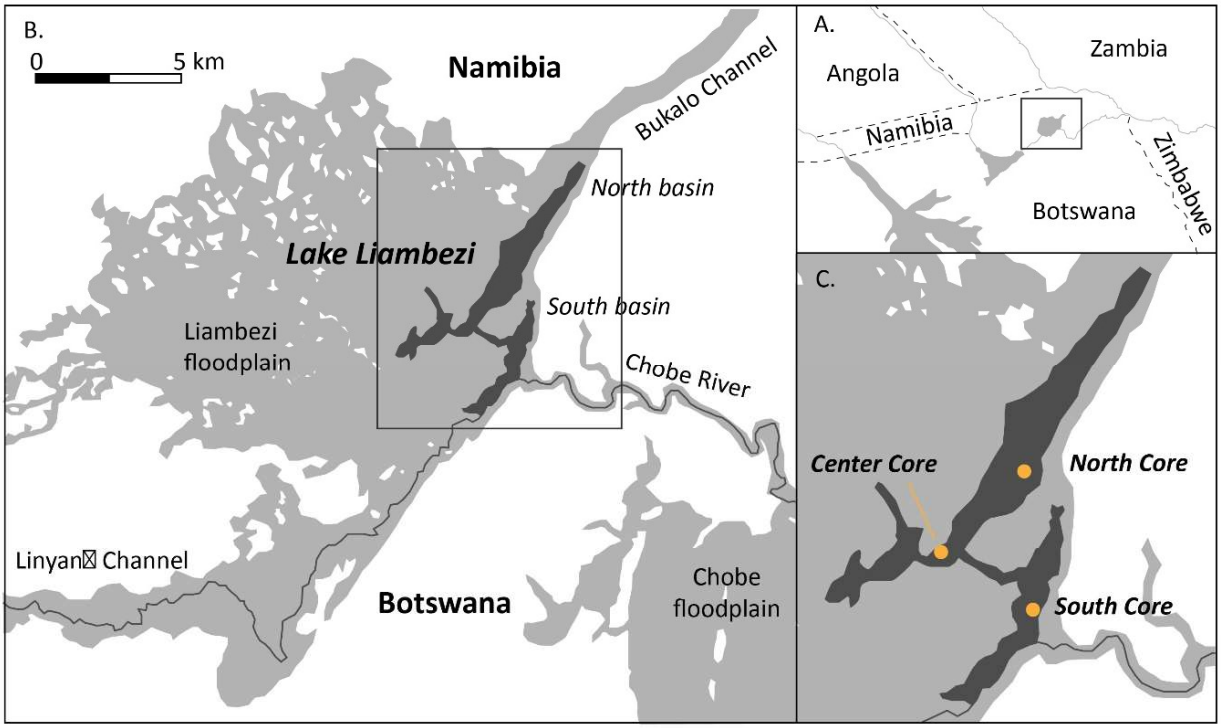

Figure 1: Map indicating (A) the location of Lake Liambezi at the border between Namibia and Botswana, (B) the lake and its surrounding environment and tributaries, and (C) the sampling sites.

Depending on the water level, the Chobe River serves as outflow of the lake (Seaman et al., 1978; Peel et al., 2015). The lake is surrounded by a major flat wetland system characterized by woodlands, wetlands and slow-flowing floodplain rivers (Seaman et al., 1978; Peel et al., 2015). Seaman et al. (1978) report a system covering some 300 km^2 of which 100 km^2 is open water at its full size. The lake changes shape, size and depth between and within years due to the source and the amount of water in the basin (Tweddle et al., 2011). The average depth is of approximately 2.5 m (Seaman et al., 1978) but can reach 7 m at the height of the rainy season (Peel et al., 2015). The general shape of the lake varies depending on the water supply. It is generally separated in two main basins: Northern and Southern basins, which are connected by a narrow 1.3 km long central channel. The lake is surround

by the Linyanti Marsh all along its western line. The Linyanti Marsh is the result of a geological fault that forms an area of wetlands composed by a complicated patchwork of swamps and marshes also known as Linyanti River. Its distance to Lake Liambezi is about 60 km south-west. The Northern sub-basin is approximately 6 km long and 1 km wide at its maximum. It receives water from the Bukalo Channel on the north, and from the channel between the two main basins on the east. All western and southern borders are supply by water from the Linyanti Marsh. The southern sub-basin gets water from multiple sources: the channel connecting the two sub-basins on the west, the Chobe River on the east, the Linyanti Marsh along its southwestern border, and the Linyanti channel at on its south-eastern border. Length and width of the Southern sub-basin are 4 km and 500 m at its maximum, respectively.

4.3.2 Sampling

Three cores were obtained from the same number of sampling locations (Figure 1C). Sampling was performed with an Uwitec Hammer Action Corer with PVC tubes of 86.0 mm inside diameter. Samples from microbiological studies were obtained under a controlled atmosphere using a Bunsen burner to sterilize all the equipment used. Samples were stored and transported in sterile plastic bags and kept refrigerated. The center core (CC) was obtained in the northern basin, at the eastern side of its southern sub-basin. It was taken about 300 m from the mouth of the connecting channel and about 500 m from the northern sub-basin. The south core (SC) was sampled at the southern part of the Northern sub-basin, about 250 m from the Chobe mouth and roughly the same distance from the Linyanti main channel. The north core (NC) was obtained in the northern basin, at the southern side of its northern sub-basin. The distance to the Bukalo Channel is about 4.2 km and to the southern sub-basin about 1.3 km.

4.3.3 Sedimentology

A number of complementary methods were used for the characterization of the sediments. Particular emphasis was placed on the analysis of clay minerals as a proxy to the climatic conditions established during the deposition of the sediments. After detailed scanning electron microscopy investigations, the amount and type of organic matter was measured using an elemental analyser (hydrogen and nitrogen) and Rock Eval studies (maturity, type and origin of organic matter). The total carbon content and stable carbon isotope compositions of the organic matter were also analyzed. The hydrodynamics of the fluviatile or lacustrine system was evaluated via grain size analyses.

The content and the nature of organic carbon (and mineral carbon) was analyzed using a Rock-Eval 6 pyrolyzer (Vinci Technologies, Rueil-Malmaison, France), as described in Sebag et al. (2018). Total organic carbon and nitrogen were analyzed using the Thermo Finnigan Flash EA 1112 analyzer. Samples were heated up to 500 and then 900 °C in the presence of O_2, the released gas (H_2O, N_2, CO_2 an SO_2) transported over a chromatographic column and then measured by a thermal conductivity detector. Thermo Finnigan Flash EA 1112 linked to a Delta V mass spectrometer was used for the carbon isotope analyses of bulk organic matter in sediments. The sediment was combusted in the presence of O_2 and the CO_2 produced was introduced into the mass spectrometer with He as the transporting agent. Grain size was determined using the Malvern Mastersizer 2000 grain sizer. Two modules of dispersion were used: Hydro 2000S, for wet samples and Scirocco 2000 for dry samples. XRD analyses of the whole rock was done with an ARL Thermo X'tra powder diffractometer. The samples were prepared following the

procedure of Kübler (1987) and Adatte et al. (1996). Whole-rock major element contents were determined by X-ray fluorescence (XRF) using a wavelength-dispersive PANalytical AxiosmAX spectrometer fitted with a 4 kW Rh X-ray tube. The analyses were performed on fused-disks prepared from 1.2 g of calcined sample powder mixed with Lithium-Tetraborat (1:5 mixture). The XRF calibrations were based on 21 international silicate rock reference materials. The data were reported on a loss of ignition (LOI)-free basis. Standard deviations were as follows: SiO2 0.08%, TiO2 <0.01%, Al2O3 0.02%, Fe2O3 0.01%, MgO 0.01%, CaO 0.01%, K2O 0.01%. Trace elements analyses were conducted on disks obtained by pressing 12 g of sample powder on a support of Hoechst-wax-C. The trace element calibrations were based on synthetic standards and international silicate rock reference materials. Standard deviations were as follows: Cr 0.6%, Mn 0.3%, Co 1.4%, Cu 0.6%, Zn 0.6%, Sr 0.5%, Zr 0.3%, Ba 2.1%, Pb N.A., S 0.01%. The method is based on Potts (1986).

4.3.4 Radiocarbon dating

Three to four samples per core were used for radiocarbon dating. Small pieces of charcoal (macro rest) were handpicked in both the central and northern cores due to the large amount of organic matter rests in the sediment. Due to the absence of such elements in the southern core, for the latter, bulk material was directly sent to the Laboratory of Ion Beam Physics at the ETH Zürich. The samples were prepared according to the methods described in Hajdas (2008) and Hajdas et al. (2007) and the age calculated following the convention of Stuiver and Polach (1977) and the program OxCal 4.3 (Ramsey, 2003), using the IntCal 13 calibration curve (Reimer et al., 2014).

4.3.5 DNA extraction

DNA from both the lysis-resistant cell fraction and the total community were obtained using an indirect DNA extraction method previously described by Wunderlin et al. (2013). This method consists of a pre-extraction of cells from the sediment prior to DNA extraction. For the lysis-resistant fraction, a spore separation step was applied prior to DNA extraction (Wunderlin et al., 2014b, 2016). Briefly, the pre-extraction of cells from the sediment was done using 3 g of wet sediment added to 15 mL of Na-Hexa-meta-phosphate. The sediment slurry was homogenized using an Ultra-Thurrax® Tube Drive control (IKA, Stauffen, Germany) for 2x1 min at 15'500 rpm, followed by 10 min of sedimentation. The supernatant was retrieved and reserved for following steps, and the remaining pellet was re-extracted using the same amount of Na-Hexa-meta-phosphate (15 mL), followed by homogenization and sedimentation. The two supernatant solutions were pooled and slowly centrifuged at 20 g for 10 min to remove the remaining mineral particles, and finally filtered onto a sterile 0.2 um pore-size nitrocellulose filters (Merck Millipore, Darmstadt, Germany).

Half of the filter was used for the DNA extraction of the total bacterial community. The FastDNA®SPIN kit for soil (MP Biomedicals, USA) was used following the manufacturer procedure, with a modification consisting in three successive bead-beating steps applied in order to retrieve DNA from the hard-to-break bacterial cells (Wunderlin et al., 2013). The supernatant obtained from these three successive bead-beating steps was treated separately and pooled by ethanol precipitation at the end of the extraction. DNA extracts were resuspended in PCR-grade water. The second half of the filter was used for the DNA extraction of the lysis-resistant cell fraction, using a method for the physical separation of spores (or spore-like structures) from the vegetative cells (Wunderlin et al., 2014b, 2016). After addition of physiological water and homogenization with vortex, the resuspended samples were

heated at 65°C for 20 min, followed by two successive chemical treatments first with lysozyme (10 mg/mL) for 60 min, and then with a mix of NaOH 0.5 N and SDS 1% also for 60 min. DNase digestion was performed at this stage in order to avoid any contamination by traces of free DNA from vegetative cells. Finally, the lysis-resistant cells were retrieved on a 0.2 um pore-size filter. DNA extraction was performed using the FastDNA®SPIN kit for soil (MP Biomedicals, USA) as described above, following the same modified protocol with three successive bead-beating steps. DNA quantifications were performed using the Qubit® dsDNA HS Assay Kit, on a Qubit® 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA).

4.3.6 Sequencing and data analysis

DNA extracts were sent for sequencing to Fasteris (Geneva, Switzerland), using the Illumina MiSeq platform (Illumina, San Diego, USA). The hypervariable V3-V4 regions of the 16S rRNA gene were targeted using the universal primers Bakt_341F (5'-CCTACGGGNGGCWGCAG-3') and Bakt_805R (5'-GACTACHVGGGTATCTAATCC-3') (Herlemann et al., 2011). The Mothur toolsuite (Schloss et al., 2009) was used to analyse the sequence data, following the standard procedure of MiSeq SOP (Kozich et al., 2013), with the exception of an additional step of singleton removal applied prior to the clustering in OTUs. The alignment of amplicons and the taxonomic assignment of representative OTUs was performed using the SILVA NR v123 reference database (Quast et al., 2013). After quality filtering and removal of chimeras, a total of 7'022'420 amplicons were retained (1'565'908 unique sequences). Singletons (1'364'141 sequences) as well as unclassifiable sequences and sequences belonging to undesirable lineages (chloroplast, mitochondria, archaea, and eukaryote; corresponding to 196'256 total and 4'460 unique sequences, respectively) were also removed. Clustering of the 5'462'023 remaining sequences (197'307 unique sequences) into OTUs using average neighbor clustering and a 97% identity threshold led to the identification of 34'159 OTUs. Sequences are available in GenBank under the BioProject accession numbers PRJNA 396429.

4.3.7 Statistical and multivariate analysis

Statistical and multivariate analysis on the community and environmental data were performed using R version 3.5.1 (R Core Team, 2014), and the package *vegan* (Oksanen et al., 2017) and *phyloseq* (McMurdie & Holmes, 2013). Community structure was analyzed using principal coordinate analysis (PCoA), based on Bray-Curtis dissimilarity and Hellinger transformation of the OTU table. OTUs accounting for less than 100 sequences in the whole dataset were removed prior to the analysis, to limit the random effect of the detection of rare OTUs and, in the case of the lysis-resistant community, to reduce the potential background of OTUs representing contamination by members of the non-lysis-resistant community. The procedure was repeated for each subset of data, when analyzing single cores or community fraction. Grouping of samples was tested using hierarchical cluster analysis using the Ward algorithm based on Bray-Curtis dissimilarity, and the best number of clusters was defined using the silhouette width measure with the function *silhouette* from the *cluster* package (Maechler et al., 2019). Constrained hierarchical clustering was performed using the chclust function from the *rioja* package (Juggins, 2019). Data were transformed as described above. Environmental data were analyzed by principal component analysis (PCA) after standardization of the variables (zero mean and unit variance). Difference between groups, based on community composition, geochemical data or visual criteria, was tested using Permutational Multivariate Analysis of Variance (PERMANOVA) using the *Adonis* function from the *vegan* package, based on the same dissimilarity matrix as described

above, with 1000 permutations. Post hoc analyses for pairwise comparisons were performed using the function *pairwise.adonis* from the package *pairwiseAdonis* (Martinez Arbizu, 2017), with 1000 permutations and Holm correction. The contribution of OTUs to the variance between groups was tested using SIMPER analysis (*vegan*).

Non-exhaustive selection of sulfur-oxidizing and sulfate-reducing bacteria was performed by selecting OTUs assigned to the Family Chlorobiaceae, Chromatiaceae and Ectothiorhodospiraceae. In addition, all OTUs assigned to a genus containing the prefixes “Desulf” and "Sulf", or the expression "thio" were also selected. The genera *Rheinheimera* and *Nitrosococcus* were removed since they are not known to oxidize sulfur (Brenner, Krieg & Staley, 2005; Hayashi et al., 2018). Non-exhaustive selection of thermophilic bacteria was performed by selecting OTUs assigned to a genus containing the expression "therm". In addition, the genus *Alicyclobacillus*, whose known representatives are thermophilic or moderately thermophilic (Schleifer, 2009), and the genera *Desulfurispora* and *Desulfotomaculum*, both including thermophilic representatives (Kaksonen et al., 2007; Schleifer, 2009), were included to the selection since they were found in high relative abundance in many of the samples.

4.4 Results and Discussion

4.4.1 Description of the individual sites

North core

The north core (NC) was retrieved in the middle of the northern basin in the deepest part of that basin, and was 35.5 cm long. The core was sub-sampled in 11 samples, based on its visual characteristics when opened (Figure 2). Samples were then grouped in three sections due to similar characteristics of sediments. The first section included samples NC15 to NC23 (21-32 cm). Sediments showed a diffuse shiny black lamination, with a clayey texture in very compact aggregates. The structure was compact, with plasticity, sticky and poorly friable. The sediment had a strong smell of organic matter and plant macro-debris were absent (which was also the case in the other sections). The second section included samples NC04 to NC14 (4-19 cm). Like the preceding section, sediments showed a diffuse shiny black lamination and a clayey texture in very compact aggregates. In contrast to the preceding section, the sediment had a loose structure with no plasticity, and was not sticky but friable. The smell of organic matter was less marked. The third section included only sample NC02 (1 cm). This horizon showed a diffuse shiny black lamination as well. Texture was fine clayey with a loose structure. Plasticity was high and sediment was sticky and friable.

In the lower section of the core (NC23-NC15), sediments were characterized by low organic carbon (C_{org}) and nitrogen (N_{org}), high C/N ratio, mid $\delta^{13}C$, mid OI, low HI, and higher proportion of sand in the grainsize (Figure 2). The characterization of the organic matter based on C/N ratio and $\delta^{13}C$ indicates a lacustrine origin of the organic matter (Figure 5A). The quality of the organic matter, based on the HI-OI indices from the Rock Eval analysis, suggests better oxidation of the organic matter compared to the overlying sections (Figure 5B). In addition, the low C_{org} also suggest higher degradation of the organic matter. The shift to a higher T_{max} in sample NC20 might reflect the higher resistance of siliceous algae to degradation (diatoms) and indicates higher lacustrine productivity. The following section of the core (NC04-NC14) brings a change in the deposits. The sharp increase in organic carbon and the shift in the quality of the organic matter suggests an accumulation and a better preservation of the organic matter. The smaller grainsize also supports low energy conditions typical for a higher water stand. The shift to the following section was already observed in sample NC04, which exhibits

intermediate characteristics, illustrating a gradual rather than an abrupt change in the environmental conditions. In the upper section (sample NC02), C_{org} remains relatively constant, while C/N ratio and $\delta^{13}C$ sharply decrease, and N_{org}, OI, HI and T_{max} slightly increase. This indicates low oxidation of the organic matter and a decrease of the terrestrial organic matter input.

The geological data was compared to the characterization of the bacterial communities from the different sediment layers. In each layer, the total community and the lysis-resistant community were investigated. However, in several cases the analysis failed for one or the other and therefore, not a direct comparison was possible in all the cases and this was the case for the three sediment cores (samples indicated by a shade of grey in Figures 2-4). A constrained cluster analysis was performed to define groups of communities with similar composition within each core and the best number of clusters was determined using silhouette width measure. Globally, the grouping of samples was coherent with the visual and geochemical characterization of the sediments for both the total and the lysis-resistant communities. This was the case not only for the north core, but also for the center and south cores. In the north core, slight differences raised from the different clustering analyses applied to the bacterial community composition (BCC). While both samples NC14 and NC04 were assigned to the 2nd group based on the visual characteristics, analysis of the BBC placed them in the 1st group (lower section) and the 3rd (upper section) respectively, for both communities (sample NC04x was missing; Figure 2).

In the lower section (NC23-NC14), the total community was mainly composed of *Comamonas*/Comamonadaceae, BSV26 (Ignavibacteriales), Acidobacteria, Betaproteobacteria, Chloroflexi, *Thiovirga* and *Sulfuricurvum* (Figure 2; Supplementary Table 1). The five last taxa were also identified as representatives of this section by the SIMPER analysis (Supplementary Table 4). The 2nd section (NC11-NC06) saw an Increase in *Acidovorax*, *Thiobacillus*, *Comamonas*, *Hydrogenophaga*, *Pseudarcicella*, as indicated by Simper analysis. Other abundant taxa were *Novosphingobium*, Comamonadaceae, BSV26 (Ignavibacteriales). The section was also marked by the almost disappearance of *Sulfuricurvum*, *Thiovirga*, and *Alicyclobacillus.* The total community of the upper most section of the core (NC04 and NC02) was characterized by an increase in organisms associated to the superphylum Parcubacteria, and the orders Burkholderiales and Xanthomonadales. Most abundant taxa also included representatives of Anaerolinaceae, Ignavibacteriales (BSV26), Betaproteobacteria, Chloroflexi, and Bacteroidetes. A sharp decrease in *Thiobacillus* was also noted (disappearing in sample NC02). In the lower section of the core (NC23x-NC14x), the lysis-resistant community appeared to be dominated by *Arthrobacter*, which could represent almost 50% of the community. Other abundant taxa were Clostridia, and *Alicyclobacillus*, while SIMPER analysis identified Acidimicrobiales as an indicator of this core section. The middle section (NC1x1-NC06x) saw the decrease of *Arthrobacter* compared to the preceding dry period, notably in samples NC06x and NC08x. The section was also characterized by *Comamonas*, *Hydrogenophaga* and other Comamonadaceae, *Acidovorax*, *Novosphyngobium*, DA111 (Rhodospirillales), *Thiobacillus*, *Desulfurispora* and *Alicyclobacillus,* as indicated by their relative abundance and the SIMPER analysis. In the upper section (represented only by NC02x since NC04x was missing), an increase in *Clostridium*/Clostridiacae, Peptostreptococcaceae and Anaerolinaceae, and a decrease in *Alicyclobacillus* was observed. *Bacillus* and Chloroflexi were also among the most abundant taxa.

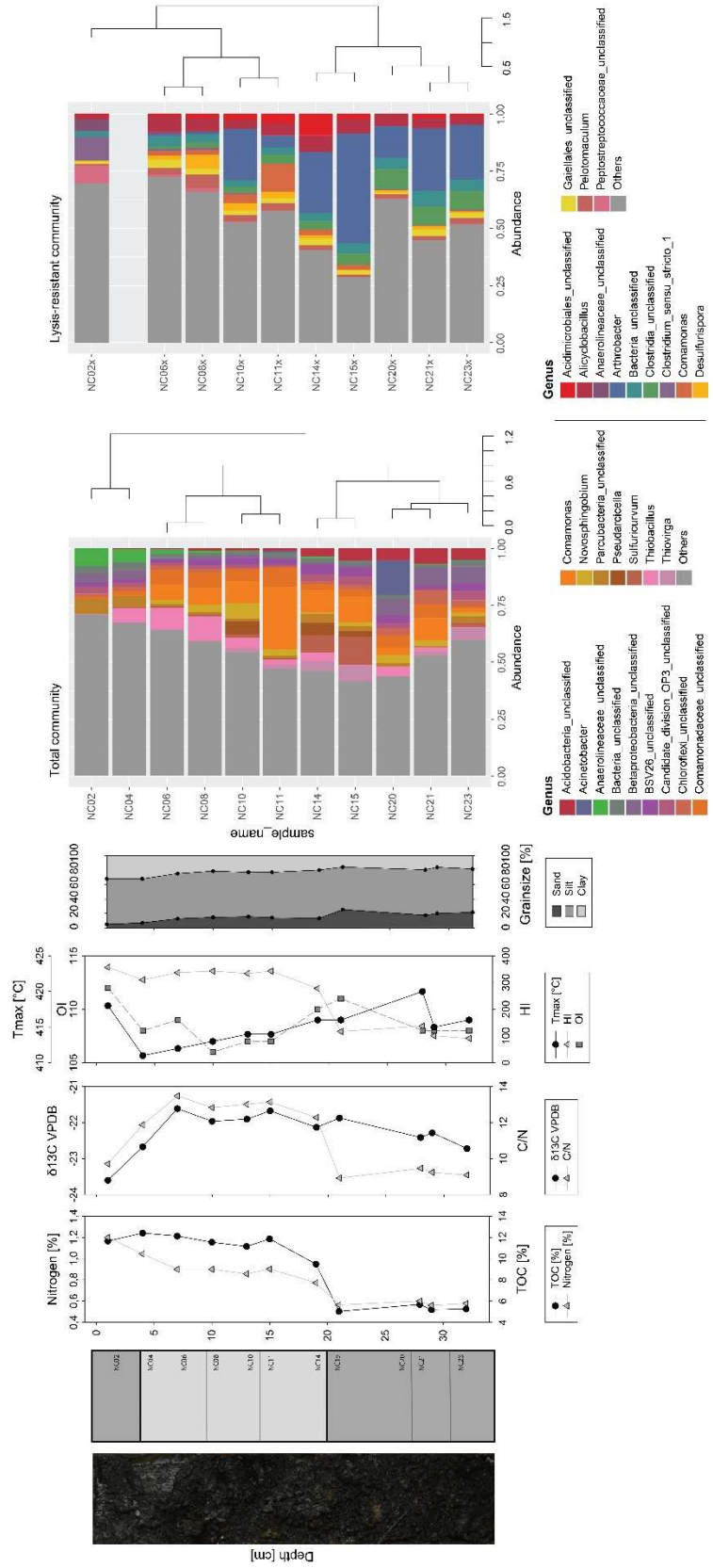

Figure 2: Description of the north core (NC) including total organic carbon (TOC=Corg) and nitrogen (Norg) from the CHN analysis, C/N ratio, δ13C, HI-OI indices and Tmax from the Rock Eval analysis, granulometry and the characterization of the total and lysis-resistant bacterial communities, based on 16S rRNA gene amplicon sequencing. Only the most abundant genera (>2% of the community in average or >5% in one sample) are shown. Constrained hierarchical clustering was performed using the chclust function based on Bray-Curtis dissimilarity and the Hellinger-transformed community table. For each community, only the OTUs with at least 100 sequences among all samples were kept.

Center core

The center core (CC), which was obtained near the mouth of the connecting channel, on the edge of the north basin, where water depth was lower. The core had a total length of 41 cm and was the longest of the cores obtained. Based on the visual characteristics of the sediment, 12 different sub-sampling were made (Figure 3), that were further grouped into four sections (or groups). The 1st group includes samples CC20 to CC29 (26-39 cm). The sediments were very fine-laminated, with a diffuse horizontal orientation, and a matte black color. Texture was clayey in fine lamination and the structure was very compact, with no plasticity. Sediments were not sticky and cohesive. Organic matter smell was light. No macro-debris of plants were observed. The 2nd group includes samples CC12 to CC17 (19-23 cm). Except a change in color from matte black to matte brown, observations were similar to the preceding section. Only sample CC12 showed a more complex orientation of the lamination. The 3rd group includes samples CC08 to CC10 (11-14 cm). These horizons showed a heterogeneous sloping lamination, clearly marked with the overlaying horizons. Their color was grey to beige with numerous marmorization traces in sample CC10, which indicate periodic emersions and the complete drying out of the sediment. The texture was clayey. The structure was compact, and showed plasticity. Sediments were sticky and friable. No smell and macro-debris of plants were noted. The 4th group includes samples CC02 to CC05 (2-7 cm). These horizons showed a clear horizontal lamination, with matte black color. The texture was clayey, in aggregates. The sediments had a loose structure, very friable, and were slightly plastic and sticky. Organic matter smell was strong and macro-debris of plants were absent.

Sediments from the lower section of the core (CC20-CC29) were characterized by low C_{org} and N_{org} content, variable C/N ratio, high $\delta^{13}C$, and low HI and OI (Figure 3). Characterization of the organic matter indicates a mixed origin (terrestrial and lacustrine; Figure 5C). The quality of the organic matter, with low OI and HI, might testify of this mixed origin, and relative low oxidation (Figure 5D). However, low C_{org} accumulation and low HI suggest a high degradation of organic matter. Samples from the 2nd section of the core (CC17-CC12) showed high variability in their geochemical and sedimentological characteristics, although they were grouped together based on their visual characteristics. In samples CC17 and CC14, increasing C_{org} attests the accumulation of organic matter, reaching its maximum in CC14. The characterization of the organic matter demonstrated a higher contribution of terrestrial organic matter and its better preservation (increasing HI and C_{org}). Grainsize showed an increase in sand proportion, reflecting a higher water energy. The trend was inverted in the following sample CC12, suggesting a return to the preceding environmental settings. In the 3rd section (CC08 and CC10), the trend already observed in sample CC12 continued, with a decrease in C_{org} and N_{org}, C/N ratio, $\delta^{13}C$ and HI, and an increase of OI. Important oxidation of the organic matter, as illustrated by the HI and OI, and low accumulation of C_{org} supports the periodic emersion suggested by marmorization traces in sample CC10. The origin of the organic matter indicates a diminution of terrestrial contribution, probably resulting from a decrease in terrestrial productivity due to the dry climate. Increase in the grainsize attested high energy in water flow, reflecting the seasonal flooding. The absence of marmorization traces in sample CC08 suggests a water-logged environment, indicating a gradual re-flooding of the sediment resulting from a more humid climate. In the upper section of the core (CC02-CC05), a sharp increase in C_{org} and N_{org}, C/N ratio and $\delta^{13}C$, combined to a slight increase of HI and a sharp decrease of OI was observed. Characterization of the organic matter indicated its mixed origin, with an increasing contribution of terrestrial material, and low oxidation. Progressive accumulation of C_{org} indicated a better preservation and/or higher productivity. This suggests higher water inputs compared to the preceding section. The decrease in sand proportion also support an increasing lake level, leading to decreasing energy of water flow.

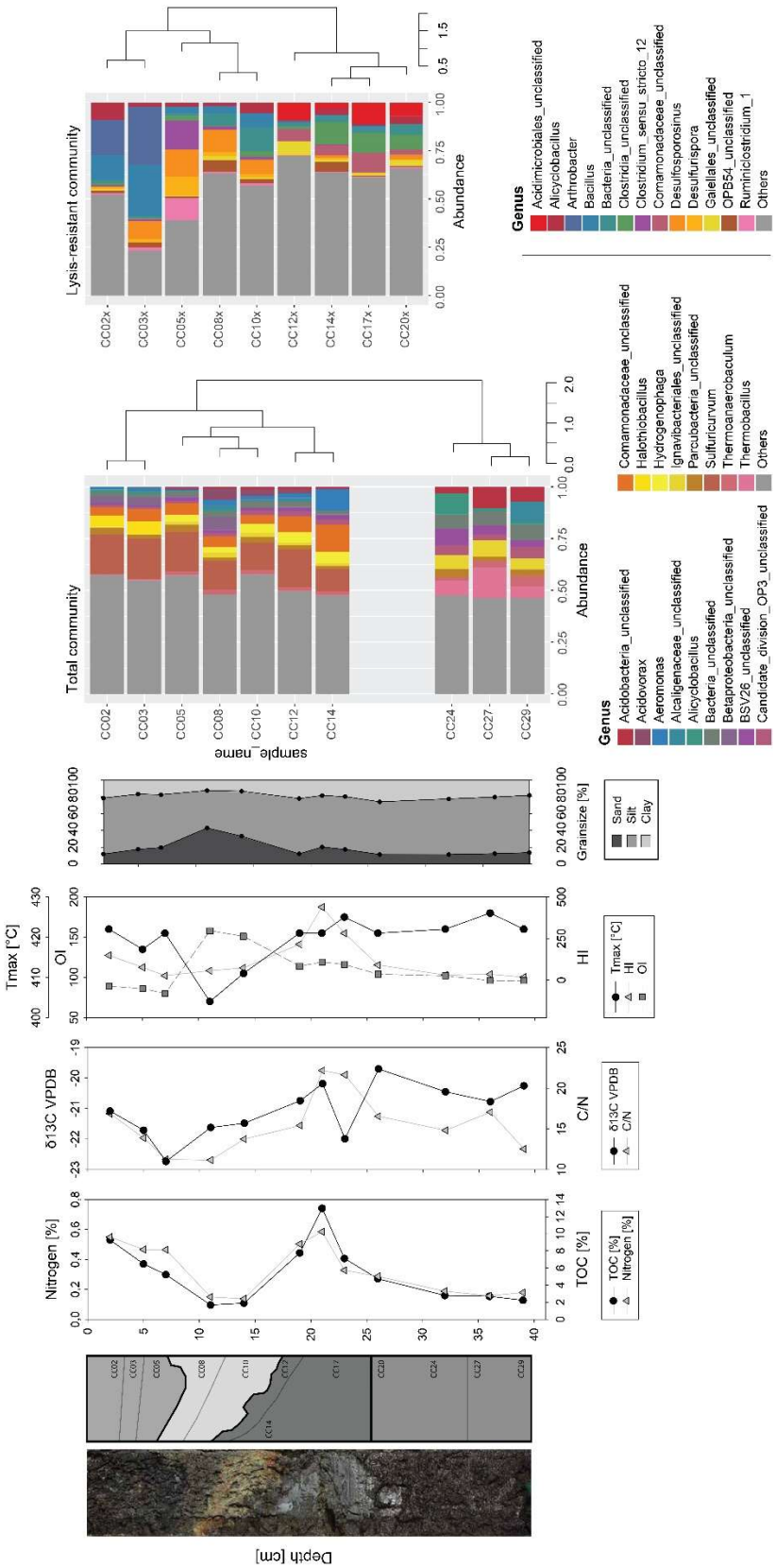

Figure 3: Description of the center core (CC) including total organic carbon (TOC=Corg) and nitrogen (Norg) from the CHN analysis, C/N ratio, δ13C, HI-OI indices and Tmax from the Rock Eval analysis, granulometry and the characterization of the total and lysis-resistant bacterial communities, based on 16S rRNA gene amplicon sequencing. Only the most abundant genera (>2% of the community in average or >5% in one sample) are shown. Constrained hierarchical clustering was performed using the chclust function based on Bray-Curtis dissimilarity and the Hellinger-transformed community table. For each community, only the OTUs with at least 100 sequences among all samples were kept.

Although the constrained clustering of the BCC was generally coherent with the visual and geochemical characterization of the sediments, some minor differences appeared. For both communities, sample CC05, assigned to the upper section based on visual characteristics, grouped with samples CC08 and CC10 (Figure 3). As well, sample CC20x clustered in the 2nd group (with CC12x to CC17x) based on the lysis-resistant community (no data for the total community), while it was assigned to the 1st group based on the visual characteristics. But this is probably due to the lack of data for the lower samples (CC29x to CC24x). A difference was also observed when comparing the cluster analyses of the total and lysis-resistant communities. For the lysis-resistant community, the main split in the clusters occurred between samples CC10x and CC12x. Although a change was also observed in the total community at this stage, it did not define two separate clusters between samples CC05 to CC14.

In the 1st section of the core (CC29-CC24), sediments exhibited high relative abundance of OTUs associated to thermophilic organisms including *Thermobacillus*, *Thermoanaerobaculum*, *Alicyclobacillus* (Figure 3, Supplementary Table 2). All these organisms have been previously isolated from hot springs or geothermal areas (Schleifer, 2009; Losey et al., 2013; Kim et al., 2014; Sahay et al., 2017). Ignavibacteriales and Acidobacteria appeared to be characteristic of this core section, as indicated by SIMPER analysis (Supplementary Table 5), and a genus belonging to the class Dehalococcoidia was among the most abundant genera. The 2nd section (represented only by CC14 and CC12) was dominated by *Sulfuricurvum.* We also observed a decrease in thermophilic and strictly anaerobic organisms compared to the preceding period (*Thermobacillus*, *Thermoanaerobaculum*, *Alicyclobacillus*, Ignavibacteriales) and an increase in aerobic (Comamonadaceae), microaerobic (*Sulfuricurvum*, *Hydrogenphaga*) and facultative aerobic organisms (*Aeromonas*). In the 3rd section, (CC10-CC05), the total community was similar to the preceding section, as indicated by the clustering analysis. However, a slight increase of *Acidovorax* and decrease of Comamonadaceae was observed. In the upper section (CC03 and CC02), similarly to the previous period, the total community was characterized by a high relative abundance of *Sulfuricurvum*, but also by the presence of other sulfur-oxidizers (*Halothiobacillus*, *Thiobacillus*, *Thiofaba*, *Alicyclobacillus*). Representatives from the Comamonadaceae, Anaerolineaceae, and Nitrospiraceae/Nitrospirales, microaerophilic, fermentative, and nitrifying (aerobic) bacteria respectively, were also among the most abundant taxa.

No data were obtained for lysis-resistant community for CC29 to CC24 (1st section). In the 2nd section (CC20x-CC12x), Acidimicrobiales, Clostridia, Comamonadaceae, *Hydrogenophaga*, *Aeromonas*, and JG37-AG-4 (Chloroflexi) were the main components of the community as indicated by their high relative abundance and SIMPER analysis. The following section (CC10x-CC05x) was characterized by an increase in *Bacillus*, Gallionellaceae, Peptococcaceae, and an unclassified Firmicutes (class OPB54), according to SIMPER analysis. The upper section was characterized by a wide domination of *Arthrobacter* and *Bacillus*, commonly found in soils, as well as the presence of several thermo-acidophilic bacteria, including *Alicyclobacillus*, *Sulfobacillus*, *Acidothermus* and *Pullulanibacillus*, previously found in hot springs and/or acid mine drainage (Mohagheghi, Grohmann & Himmel, 1986; Schleifer, 2009; Pereira et al., 2013; Méndez-García et al., 2015). The sulfur oxidizer *Desulfosporosinus* was also present in high abundance in sample CC03x.

South core

The south core (SC) was retrieved in the middle of the southern basin, close to the Chobe mouth and the Linyanti main channel (about 250 m), and was 34.3 cm deep. The core was subsampled in 15 samples that were further grouped in 2 sections (Figure 4). The first section includes samples SC17 to SC23 (25-33 cm). These horizons showed a diffuse, dark brown, shiny horizontal lamination. The

texture was clayey. The sediments were very compact and showed great plasticity. There were non-sticky but cohesive. Strong smell of organic matter and presence of macro-debris of plants were noted. The second section includes samples SC01 to SC16 (0-23 cm). These horizons showed a clear horizontal lamination, of shiny black color. The texture was clayey in fine lamination. The sediments were very compact and showed great plasticity. They were sticky, slightly friable, and emitted a strong smell of organic matter. No macro-debris of plants were observed.

In the lower section of the core (SC17-SC23), sediments showed relative constant characteristics from sample SC23 to SC 19, followed by a progressive change in sample samples SC18 and SC17. Samples SC23 to SC 19 showed low C_{org} and N_{org}, and high C/N ratio and $\delta^{13}C$ (Figure 4). Characterization of the organic matter based on C/N ratio and $\delta^{13}C$ demonstrated a mix between a terrestrial input and a local algae production (Figure 4E). Quality of the organic matter, as shown by the Rock Eval analysis, revealed high OI and T_{max}, and low HI, and testified of a higher oxidation compared to the overlying samples (Figure 4F). This suggests a higher degradation of organic matter, which is consistent with the low accumulation of C_{org}. High T_{max} might reflect the contribution of siliceous algae. The higher proportion of sand in these samples suggests a higher water energy, perhaps related to a decrease in the water levels and hence increasing fluviatile conditions. An increase in both C_{org} and HI in samples SC17 and SC18 indicated accumulation and preservation of organic matter. The origin and quality of the organic matter did not show a significant change, although the decrease of T_{max}

could reflect a higher proportion of terrestrial inputs. In upper section of the core (SC01-SC16), the trends observed in the previous samples SC17 and SC18 were more pronounced. $C_{org,}$ N_{org} and $\delta^{13}C$ fixed to high and low values respectively, while C/N ratio decreased progressively. Rock Eval analysis showed irregular decrease of OI and increase of HI and T_{max}. High C_{org} indicated a higher accumulation of organic matter. Its characterization revealed a shift in its origin and quality. Mainly from lacustrine origin, as indicated by the relation between C/N ratio and $\delta^{13}C$, as well as the increasing T_{max}, the organic matter showed a low degree of degradation (high HI and low OI), which is consistent with the increasing C_{org} accumulation. In addition, the decreasing grain size suggest decreasing water energy, and would support the hypothesis of an increasing lake level and the establishment of lacustrine conditions.

Total community in the lower section of the core (SC17-SC22) was highly dominated by *Thiobacillus* and to a lesser extent *Sulfuricurvum* (sulfur-oxidizing bacteria; Kodama and Watanabe 2004; Sievers and Swings 2005), which can represent almost 40% of the community in lower samples (Figure 4). Other abundant and representatives genera were *Bacillus* (aerobe or facultative anaerobe) (Schleifer, 2009), and genera belonging to the family Anaerolinaceae (fermenter) (Yamada et al., 2006), Gallionellaceae (iron-oxidizer) (Sievers & Swings, 2005), Gemmatimonadaceae and Commamomonadaceae (both aerobes) (Sievers & Swings, 2005; Krieg et al., 2010) (Supplementary Table 3 and 6). In the upper section of the core (SC01-SC16), an unclassified Anaerolinaceae was the most abundant genus. *Bacillus* was also among the most abundant taxa, although its relative abundance decreased compared to the preceding period. The total community was also characterized by an increase in Xanthomonadales, Burkholderiales and Nitrospiraceae/Nitrospirales (4-29_unclassified) abundance. Sulfur- and iron-oxidizing bacteria (*Thiobacillus*, *Sulfuricurvum* Gallionellaceae) were well detected in the first two samples of the section (SC16 and SC15) and decreased in the top of the core. Community appeared to be extremely diverse, with ~75% of the community being composed of genera accounting for less than 2% of the whole community in average, or 5% in maximum.

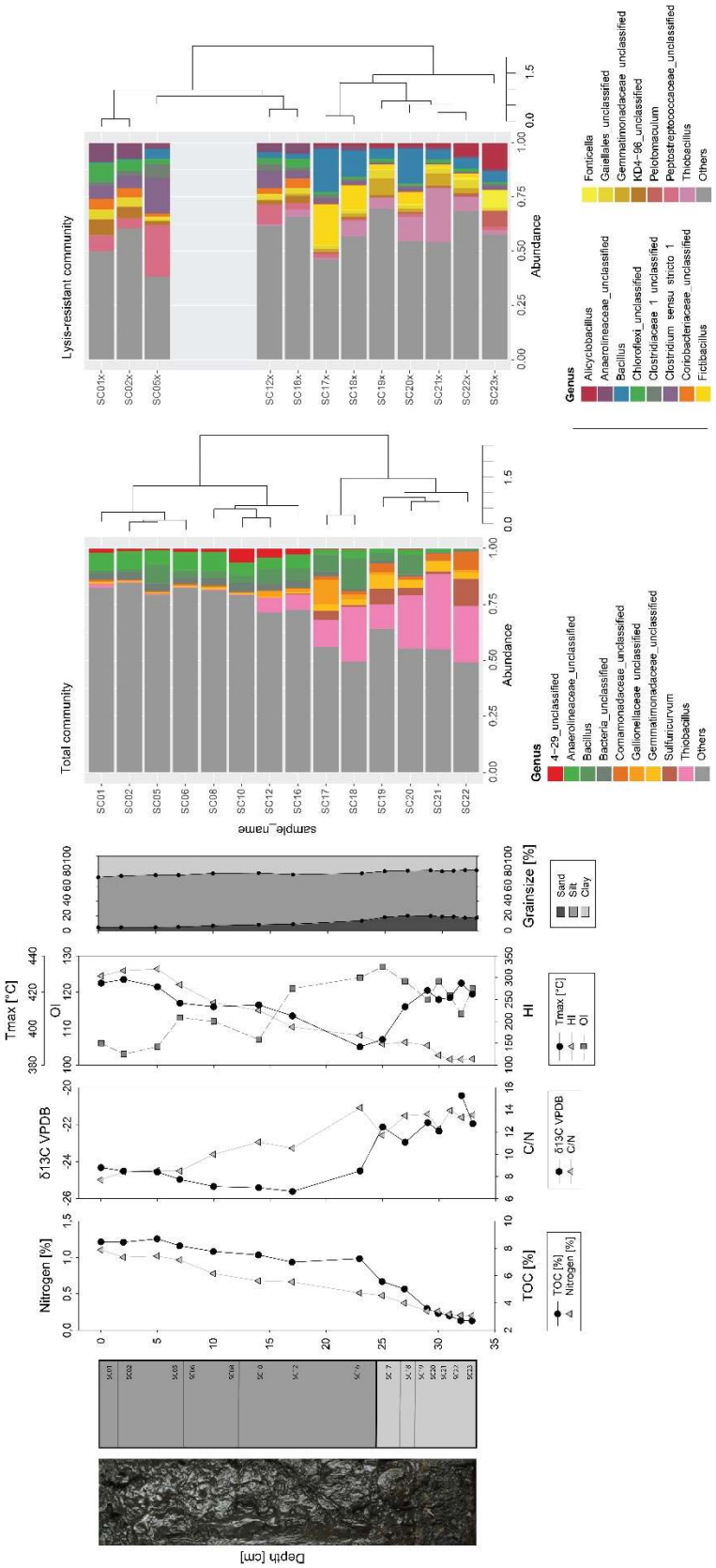

Figure 4: Description of the south core (SC) including total organic carbon (TOC=Corg) and nitrogen (Norg) from from the CHN analysis, C/N ratio, δ13C, HI-OI indices and Tmax from the Rock Eval analysis, granulometry and the characterization total and lysis-resistant bacterial communities, based on 16S rRNA gene amplicon sequencing. Only the most abundant genera (>2% of the community in average or >5% in one sample) are shown. Constrained hierarchical clustering was performed using the chclust function based on Bray-Curtis dissimilarity and the Hellinger-transformed community table. For each community, only the OTUs with at least 100 sequences among all samples were kept.

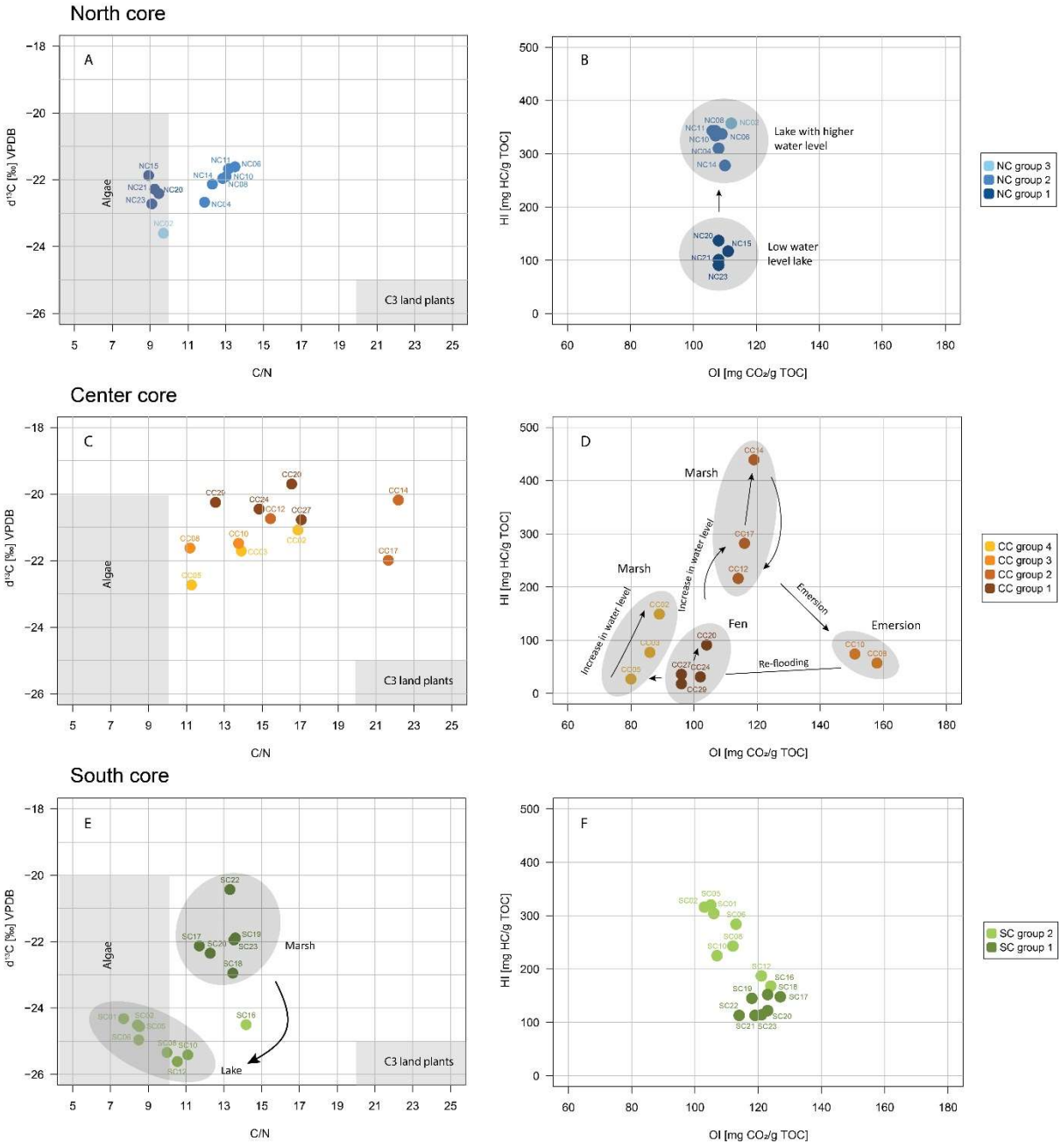

Figure 5: Characterization of the organic matter. On the left, graphics defining the origin of the organic matter, based on δ13C and C/N ratio for samples from the (A) north core, (C) center core, (E) and south core. On the right, graphics describing the quality of the organic matter, based on HI-OI indices from the Rock Eval analysis for samples from the (B) north core, (D) center core, (F) and south core.

Regarding the lysis-resistant community, the lower section of the core was characterized by a high relative abundance of *Thiobacillus*, *Bacillus* and *Fictibacillus*, with high variations between samples. Gaiellales, *Gemmatimonas*/Gemmatimonadaceae and *Alicyclobacillus* were also among the most abundant taxa, although the latter decrease along the section. Although sample SC23x belong to the same cluster, it exhibited a particular community, composed of *Alicyclobacillus*, *Pelotomaculum* (anaerobic, termite guts and anaerobic sludge), *Fonticella* (hot springs) and *Bacillus* as main representatives. In the upper section (SC01x-SC16x), the lysis-resistant community was characterized

by a sharp increase in *Clostridium*/Clostridiaceae, Peptostreptococcaceae, Coriobacteriaceae, all associated to mammal microbiome (Lozupone et al., 2012; Clavel, Lepage & Charrier, 2014; Browne et al., 2016). Likewise, Comamonadaceae and an unidentified Chloroflexi (KD4-96_unclassified) showed to increase. Gaiellales and Anaerolinaceae were also identified among the most abundant taxa. We also observed the decrease *Alicyclobacillus*.

4.4.2 Initial age model based on radiocarbon dating

An initial age model was attempted based on radiocarbon measurements for a selected number of samples (Table 1). According to these measurements, each one of the cores represented a different time period despite their similarity in length. Three of the four measurements for the center core (CC10, CC20, and CC27) were coherent and suggested that this core represented close to the last 5000 years of history of the lake. However, the measurement made in the upper most sample (CC03) was inconsistent as it corresponded to an older period of time as compared to the deeper samples, and could be caused by contamination with an older charcoal material deposited in younger sediments. A projection based on the three retained measurements resulted in a linear sedimentation rate. In the case of the north core two out of the three measurements were deemed incoherent, and therefore radiocarbon dating could not be used to create an age model. In this case also, the position of the chosen charcoal macro-rests may be the result of reworking into the sediment or a late deposit transport. Finally, for the south core, three out of four measurements were retained (SC17 was not considered due to a much younger age in comparison to overlying layers). The radiocarbon dating suggested that this core covered only the last 900 years of lake history. A projection based on the retained measurements also suggest a linear sedimentation rate, and it shows that the selection of bulk material due to the absence of macro-rest does not seem to be problematic.

*Table 1: Results of the radiocarbon dating for the 11 samples selected. An * indicates values that were not retained.*

sample	**^{14}C dating** (years BP)
NC04	1673 ± 23*
NC11	1740 ± 23*
NC23	1618 ± 23*
CC03	2710 ± 22*
CC10	2006 ± 23
CC20	3243 ± 24
CC27	4697 ± 25
SC05	259 ± 21
SC12	424 ± 21
SC17	219 ± 21*
SC22	898 ± 21

4.4.3 Producing a revised age model resulting of the analysis of the bacterial communities and geochemical indicators

Given the results of the radiocarbon dating and the initial geochemical and microbiological characterization that shows apparently a unique pattern for each individual core, at a first glance, it appears difficult to connect the three cores alongside a common timescale. However, by considering an overall clustering of the samples based on bacterial community composition, as well as by analyzing selected geochemical indicators, a revised age model connecting the three sediment sequences was generated.

4.4.4 Overall grouping of the sediment cores based on the bacterial community composition

When analyzing each core separately, the grouping of the samples based on the structure of the bacterial communities (cluster analysis), corresponded mostly to the groups identified on the basis of the visual characterization, and subsequently geochemical analyses (Figure 2-4). Although some mismatches were noted (for example, NC4, NC14, CC05), this concerned mainly transition samples where a time-lag in the response of bacterial community to the environmental change could be expected. This suggests that changes in the bacterial community composition (BCC) reflect environmental/ecological differences registered in the sediments and could be used to ameliorate the interpretation of the environmental history of Lake Liambezi. The overall clustering of the entire dataset (total community) resulted in four main groups of samples which were organized along a timeline (sediment depth) sequence (Figure 6A). The first group included the two upper samples of the north core (NC02 and NC04) and the upper part of the south core (SC01-SC16). The similarity of their bacterial community, strongly suggests they might belong to the same time period, corresponding to the last 600 years as indicated by the radiocarbon dating of the south core. The second group included the samples from the lower part of south core (SC17 to SC22) and samples CC03 and CC02, placing them between 450 and 900 years BP. This suggests that samples CC03 and CC02 were more ancient than what was indicated by radiocarbon dating and supporting the inconsistent radiocarbon measurement obtained for CC03. In addition, the results of the BCC clustering indicate that in the case of the center core, the upper most layers of sediment (past 400 years BP) were most likely lost during sampling. A third period was represented by the clustering of the other sediment samples of the north core (NC06-NC23), which are related to the second section of central core (CC05-CC14). Based on the radiocarbon dating of the center core, these samples were estimated to cover the period from 1000 to 3250 years BP. Finally, samples from the deepest section of the center core formed a fourth group (older than 3250 BP). The changes of the BCC over time were also illustrated on a principal coordinate analysis (PCoA) in which the samples were organized along a depth (time) gradient represented by the x axis (Figure 7). This indicates that the BCC follows a progressive change overtime (no resilience after perturbation), and that this change affected the three sampled areas in a similar manner. It is worth mentioning that the grouping of the three cores based on the bacterial community rather than allowing to assign an exact date to the samples, defined a common timeline sequence.

In addition to the general response of the communities to the global hydrological regime, the specific location from where the cores were retrieved had an important impact on the variability of the BCC. This was clearly shown on the cluster analysis (Figure 6) in which aside the separation between “recent” and “old” samples, within these groups, samples were grouped by location, demonstrating

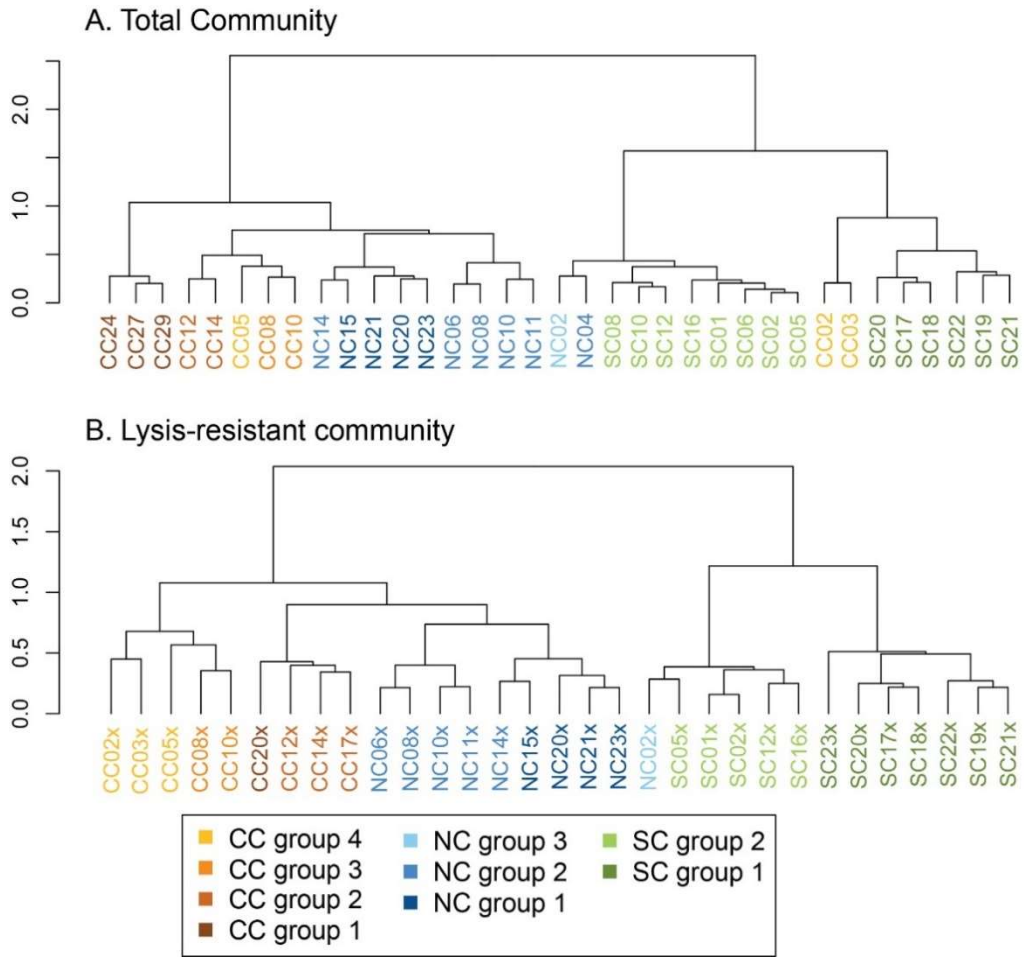

Figure 6: Clustering analysis using Ward algorithm of the (A) total community and (B) the lysis-resistant community. Distance between samples were calculated using Bray-Curtis dissimilarity based on the Hellinger-transformed community table. For each community, only the OTUs with at least 100 sequences among all samples were kept.

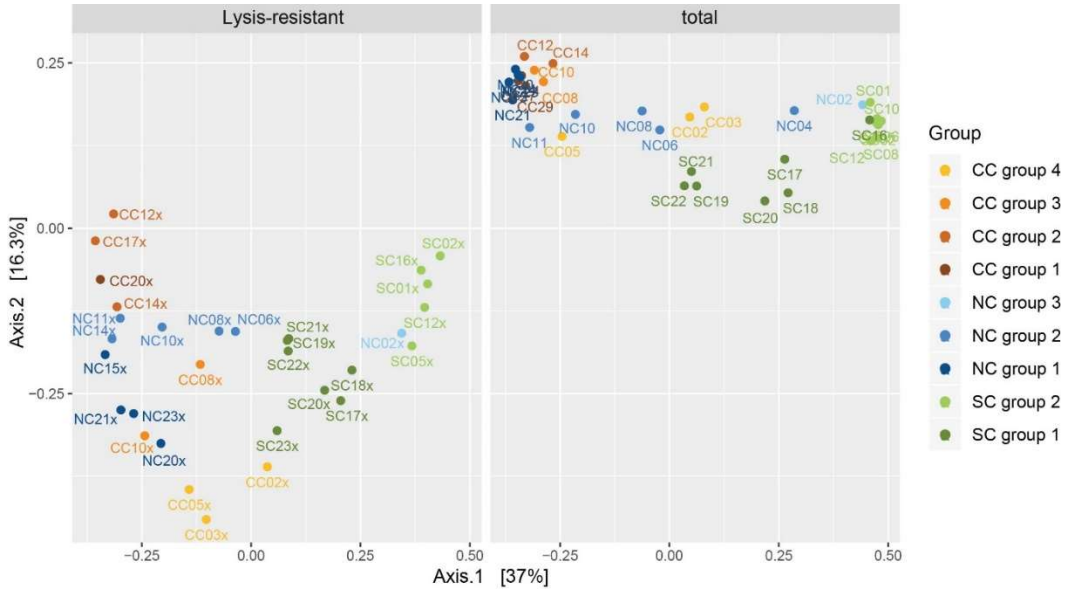

Figure 7: Principal coordinates analysis (PCoA) triplot of the total and the lysis-resistant communities based on Bray-Curtis dissimilarity and Hellinger transformation of the OTU table. OTUs represented by less than 100 sequences in the whole dataset were removed from the analysis. Colors correspond to the main grouping of samples defined for each core, identified from the visual characterization of the sediments.

the importance of the sampling site. Moreover, the apparent separation of samples from the south core on the PCoA suggests an important influence of the basin and associated watershed for this site and the existence of specific microenvironments corresponding to the sampling locations in response to variables, such as microtopography and water column depth, distance to the tributaries, and sediment deposition rate and mobility.

4.4.5 Correction based on climate-linked minerals (smectite)

Observation of the parental minerals (feldspar, plagioclase and micas) as well as precipitated clays (phyllosilicate, kaolinite and smectite) might allow reconstructing the climatic evolution of the studied site (Meunier, 2003). Weathering causes anastomosis of parental minerals as feldspar, plagioclase and micas. In contrast, crystallization of alteration minerals as clays is controlled by climatic parameters. Heavy rain all year-round favors kaolinite formation. Conversely, seasonal rains followed by intense evaporation periods conducts to soils enriched in Si, Ca and Mg. This favors the precipitation of smectite (Meunier, 2003). Following the transformation stages, phyllosilicates are observed in early stages where smectite appears in most weathered stages (Banfield & Eggleton, 1990; Meunier, 2003). All of the above parental and precipitated minerals are found in the Liambezi sediments. A PCA based on their distribution patterns indicated a common origin of feldspar, plagioclase, micas, phyllosilicate and kaolinite as detrital products (Supplementary Figure 1). Even in the case of phyllosilicates and kaolinite, which are known products of weathering, their close link with parental material in the PCA suggested a detrital origin, and transport by wind and rivers. In contrast the presence of neo-formed smectite in the Liambezi sediments seemed as a better proxy of seasonal rains followed by intense evaporation periods. Indeed, precipitation of smectite occurs during intense weathering periods (Meunier, 2003). Due to the close link between smectite and climate, smectite has been chosen as proxy to generate a climatic model with dryer (when smectite is found in lower amounts) and wetter periods (when it is found in higher amounts. Indeed, the trends between the three sites were well correlated (Figure 8).

The relative concentration of smectite was then used to adjust the age model and to propose a new sedimentation rate for each of the sites (Figure 9). Despite periods of total drying and periods of major flooding due to the monsoon and dry period in the region, the watershed as well as the amount of water for the last 8000 years was supposed to remain stable (Burrough, Thomas & Singarayer, 2009; Thomas & Burrough, 2012; Chase et al., 2015; Chevalier & Chase, 2015). If the shape of the basin did not evolve during the studied period, it can be assumed that sedimentation rate in each site was homogenous over time (Frouin et al., 2007; Bennett & Buck, 2016). The correction made with the smectite gives a sedimentation rate of 1 cm/100 years for north core and 1 cm/115 years for center core with a high stability all along the cores. Sedimentation rate in the southern core was higher with a mean value at 1 cm per 30 years.

4.4.6 Refining the interpretation of ecosystem evolution using the bacterial communities: sulfur oxidation and hydrothermalism

The clustering and PCoA analyses supported the fact that the main changes in the environmental conditions in Lake Liambezi were reflected in the bacterial community. However, it remained difficult to infer the specific drivers of this differentiation based on the analysis of the total BCC. Bacterial communities in the sediments appeared to be extremely diverse and complex (977-4766 OTUs and

214-633 genera per sample), making the interpretation of the results difficult (Wunderlin et al., 2014a). In most layers, taxa associated to various types of metabolisms (aerobic respiration, anaerobic respiration, fermentation) and environments (soil, freshwater, marsh, hot springs) were found. In addition, the taxonomic assignation of OTUs is limited at the best to the genus level, and some OTUs could only be classified to higher taxonomic level, which hinders the precise prediction of metabolic potential. Therefore, an alternative approach consisted on the selection of specific metabolisms for the inference of environmental change. The analysis of the BCC pointed towards two interesting phenomena, notably a very active sulfur cycle and hydrothermal activity.

A high relative abundance of sulfur-oxidizing bacteria was observed in most sediment layers (Figure 10A). *Sulfuricurvum*, *Thiobacullus*, *Thiovirga* were the most abundant genera, their proportions varying over time and across cores. In contrast, the relative abundance of sulfate reducers was much lower and a community of sulfate reducers was mainly seen in the lysis-resistant fraction (Figure 10B). The higher relative abundance of sulfur oxidizers compared to sulfate reducers can be explained by the usual low content of sulfate in lacustrine environments compared to marine ones (Jin et al., 2017). Sulfate reduction is often considered to be a minor metabolism in lacustrine sediments, due to low availability of sulfate and organic matter. However, in eutrophic lakes the higher productivity and the rapid consumption of oxygen might favor this metabolism (Holmer & Storkholm, 2001). High abundance of *Sulfuricurvum* and *Thiobacillus* have already been reported in eutrophic lakes (Chen et al., 2015), and thus, similar conditions might have occurred in Lake Liambezi, at least periodically, due to an increase in ecosystem productivity.

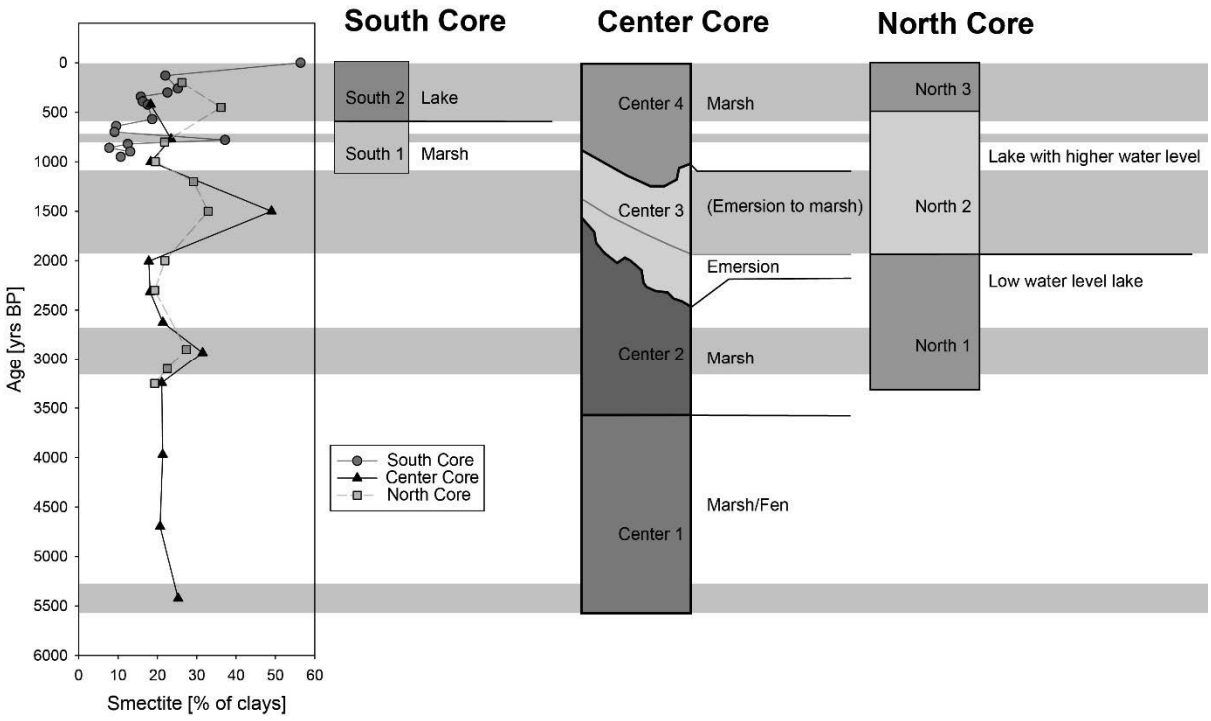

Figure 8: Climatic model, based on the age model, the evolution of the lake regime at the different location, and the smectite profiles.

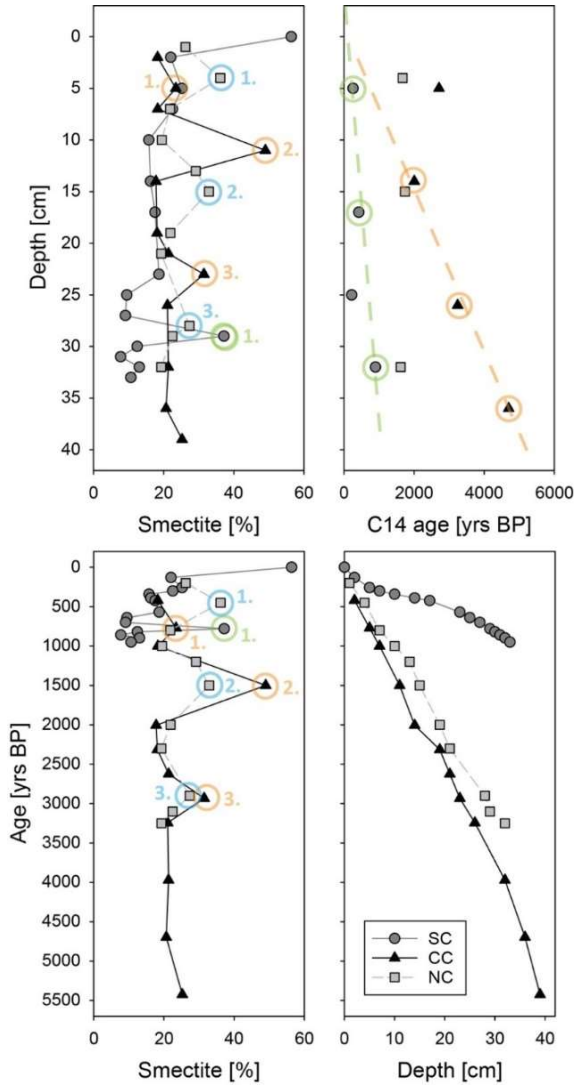

Figure 9: Calibration of the three cores (NC=north, CC=center, SC=south) and elaboration of the age model based on smectite measurements and the calculated sedimentation rate. Above, uncorrected model in function of sediment depth. Below, corrected age model.

While sulfate-reducing bacteria showed a relatively constant abundance in the total community, their abundance pattern was highly variable in the lysis-resistant community. Notably, a sharp increase in sulfate reducers was observed in the section CC10x-CC03x was observed in the center core (and to a minor extent in the north core). This was characterized by a high abundance of *Desulfurisporosinus*, *Desulfurispora* and *Desulfotomaculum* (*Desulfurispora* in the north core). The last two genera include thermophilic species (Kaksonen et al., 2007; Schleifer, 2009). Interestingly, this increase corresponds to a period when hydrothermal activity could be supposed to be intensive (see below), suggesting a link between hydrothermalism and the sulfur cycling. Detection of thermophilic sulfate-reducing bacteria has already been reported in hot sediments from a hydrothermal vent in a Lake Tanganyika, with the availability of sulfate being attributed to hydrothermal fluids (Elsgaard et al., 1994). However, an increase of sulfate-reducing organisms was not observed in the total community, which suggests that these organisms were deposited in an inactive state. The deposition of exogenous spores, originating from distant hydrothermal sources, could also partly explained the abundance patterns. However, such exogenous inputs could be expected to be more important in the proximity of an inflow, which is not the case here.

The presence of a high proportion of thermophilic and moderately thermophilic bacteria (Figure 10C) could be interpreted as evidence of hydrothermal activity. *Alicyclobacillus*, *Thermoanaerobaculum*, *Thermobacillus*, *Acidothermus* and *Desulfurispora* were among the most abundant taxa. The optimum growth temperatures for these groups ranged between 55 °C to 60 °C (Mohagheghi, Grohmann & Himmel, 1986; Kaksonen et al., 2007; Schleifer, 2009; Losey et al., 2013), except for *Alicyclobacillus* (35-65°C) (Schleifer, 2009). Interestingly, a previous study reported low temperature hydrothermal activity in Kasane (about 100 km from the sampling site), with temperatures ranging from 25°C to 50°C (Tebuho Mukwati et al., 2018). While the abundance of thermophilic bacteria in the lysis-resistant fraction could be attributed to hydrothermal activity occurring in the region and the transport and deposition of spores or lysis-resistant structures, the presence and high relative abundance of organisms with optimum temperature growth of 55-60 °C in the total fraction suggests hydrothermal activity as a relevant process happening in the lake.

In summary, the availability of organic matter/hydrothermal activity could change over time and be a driver of community selection. Therefore, abundance patterns of sulfur-oxidizing, sulfate reducing, and thermophilic bacteria could be used as a proxy to strengthen the interpretation of ecosystem history. The correlation of the abundance patterns of sulfur-oxidizers and thermophiles in the three cores illustrates the effect of the corrections made in the age and climatic models contributed by the analysis of the BCC and geochemical proxies (smectite). When the relative abundance of sulfur-oxidizers and thermophiles is displayed across a common time line for the three cores (Figure 11), the similarity of the patterns of abundance and community composition are striking. For instance, the section corresponding to the upper samples of the north core and the upper part of the south core (lacustrine environment; present to 301 years BP) was characterized by the almost entire absence of sulfur-oxidizing and thermophilic bacteria. Both groups become abundant after a transition period comprising the lower part of south core and samples CC03 and CC02 (approximately 420 to 900 years BP) and the other sediment layers of the north core and central section of the center core (around 900 to 3500 years BP). Finally, in the deepest section of the center core (covering 3900 years BP and beyond), thermophilic bacteria were highly abundant while sulfur-oxidizing bacteria were almost undetectable. These changes in relative abundance can be assumed to be the result of environmental modification impacting all the ecosystem simultaneously.

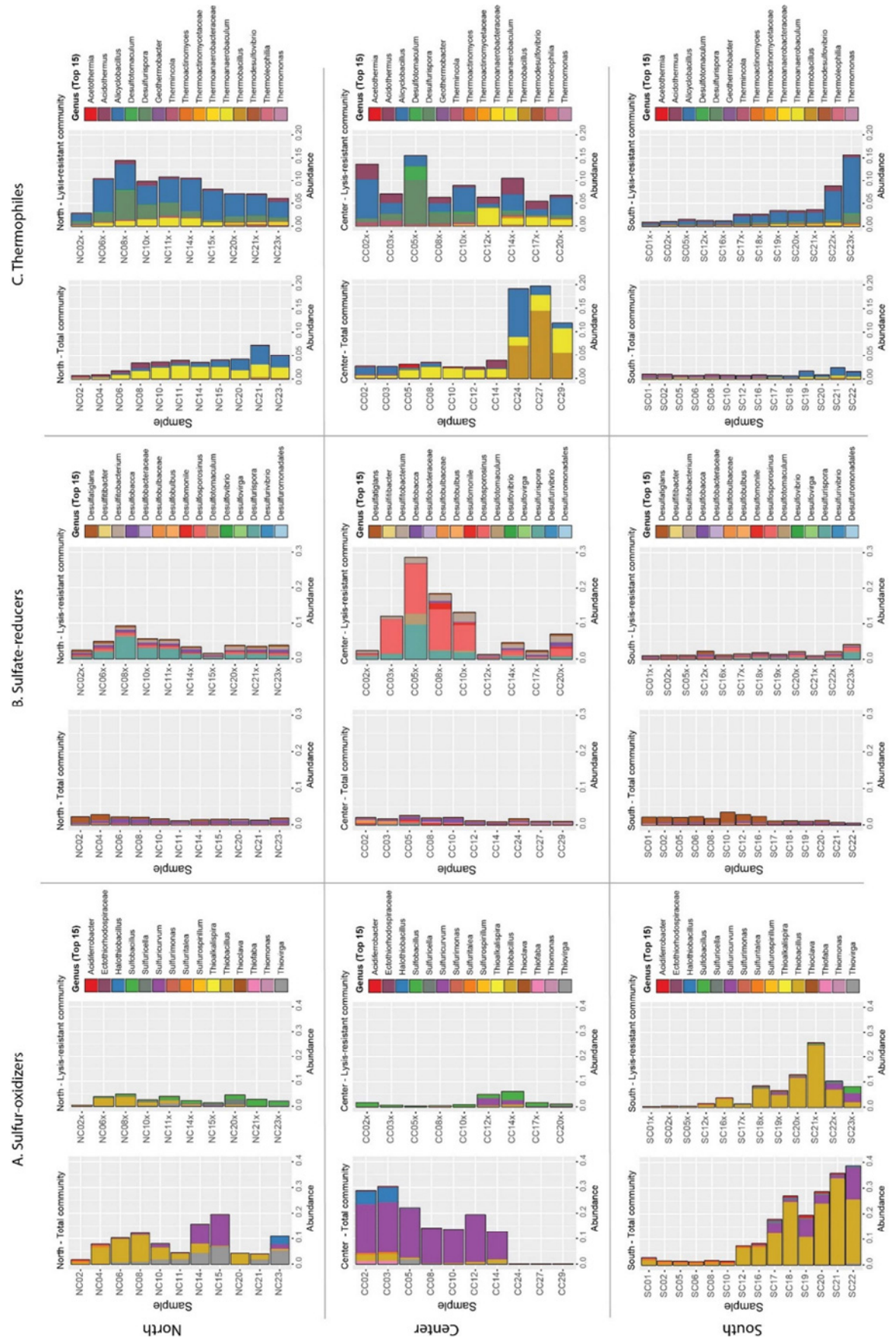

Figure 10: Characterization of the (A) sulfur-oxidizing, (B) sulfate-reducing and (C) thermophilic populations from the total and lysis-resistant community in sediments from Lake Liambezi, based on 16S rRNA gene amplicon sequencing. Relative abundance of the 15 most abundant genera mean) is represented.

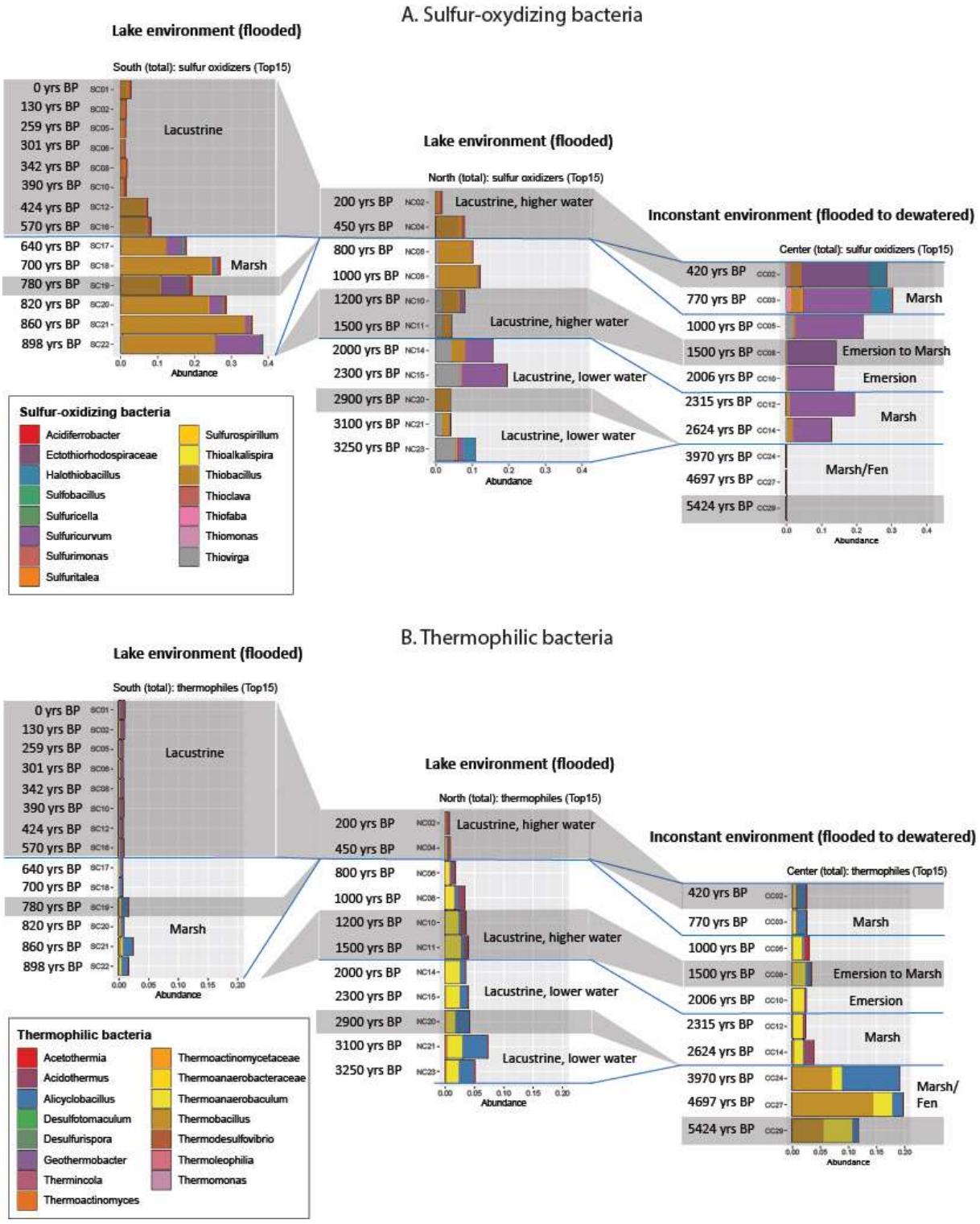

Figure 11: Calibration of the three cores and age model based on the similarities of (A) the sulfur-oxidizing and (B) the thermophilic bacteria across cores. The blue lines delimitate the groups of samples based on the clustering analysis.

4.4.7 Reconstruction of the ecosystem history

5500 to 3200 years BP (dry period)

The low abundance of smectite during the period extending from ~5000 until 3200 years BP indicates a dry period. The sediments of those horizons contained smaller quantities of organic carbon, with a C/N ratio suggesting mixed origins for the organic matter (terrestrial and lacustrine primary production). A low level of oxidation of that organic matter supports partial flooding, but constant water-saturated conditions (absence of marmorisation traces). This dry period was likely preceded by a more humid period, as indicated by the slightly higher smectite abundance and the signal of a more lacustrine-originated organic matter in sample CC29. A seasonal marsh or a fen environment is proposed to characterize the end of this first recorded transition from a humid period to a dry period.

The biological interpretation of this section is only based on the total bacterial community of the center core (based on the revised age model the other cores did not cover this period; and sequencing of the lysis-resistant community did not work). In this section the presence of a high proportion of thermophilic and moderately thermophilic bacteria (including *Thermobacillus*, *Thermoanaerobaculum*, *Alicyclobacillus*) can be interpreted as evidence of hydrothermal activity in the lake (see hydrothermal activity section below). Contrary to most other layers, this hydrothermal event was not associated to the presence of sulfur-oxidizing bacteria, which were almost entirely absent, suggesting that sulfide is not available for sulfur oxidation during this period. This can be the result of either low productivity and/or a rapid turnover of organic matter, favored by oxic conditions during this mostly dry, albeit water-logged period, thus favoring aerobic conditions in the water column and at the sediment-water interface. In addition, chemical oxidation of sulfide (Whitcomb, Delaune & Patrick, 1989; Luther et al., 2011) might limit its availability for biological sulfur-oxidation.

3200 to 2000 years BP (dry period)

The period ranging from 3200 to 2000 years BP corresponds to a dry climatic period, interrupted by a humid episode at ~3000 years BP, as indicated by the smectite profile. The humid episode recorded in the smectite profile did not impact significantly the sediment, since both the characterization of the sediment and the BCC did not show major differences. In the Northern area, the proportion of smectite in samples NC23, NC21 and NC15 supports a dry period during the time covered by this section of the core. The low accumulation of organic matter with an algal/lacustrine origin and well degraded suggest lacustrine conditions, but with a low water level, favouring the degradation of organic matter. Due to a low water level, the Bukalo channel might have arrived closer to the coring site (compared to a more humid period) as shown by a higher proportion of sand in the grainsize. The impact of the humid episode indicated by the peak in smectite abundance (samples NC20 and CC17) was different between the two sites, which reflect geomorphology and location in the lake basin. The center core is located on the edge of the North basin and its maximum depth is lower. Therefore, this area is more impacted by any change in the hydrological regime. This is reflected by a more variable stability of the organic in the core, compared to the other sampling sites (south and north core). In the centre core, the humid period translates into organic matter from a terrestrial origin, fed by high productivity of terrestrial plants. Furthermore, good preservation (high concentration of poorly oxidized Corg) of organic matter supported by the immaturity of the organic matter typical for an anoxic environment such suggests a water-logged environment. The return to a dry period is attested by the opposite characteristics in CC12 (decrease concentration of organic matter with a lesser terrestrial signature and higher oxidation). The horizon CC10 (2000 years BP) reveals the apogee of the drying out observed in the preceding dry period, with a total emersion in the central area of the Lake.

In this period, sulfur oxidizers were detected in both the north and center cores (representing up to 20% in the total community), highlighting the important of the sulfur cycle. By contrast, sulfate-reducing bacteria were detected at low abundance, suggesting that the sulfide does not originate from sulfate reduction, but rather from the degradation of organic matter. The additional input of sulfide from hydrothermal sources is also plausible. Oxic conditions at the water-sediment interface associated to a dry period and low water column levels were suggested by the presence of numerous aerobes, including: *Sulfuricurvum*, *Comamonas*/Comamonadaceae and Acidobacteria in both cores, *Thiobacillus* and *Thiovirga* in the north core, and *Aeromonas*, *Acidovorax* and *Hydrogenophaga* in the center core. Moreover, the decrease in strictly anaerobic organisms (*Thermoanaerobaculum*, Ignavibacteriales and Acidobacteria) suggests increasing oxygen availability in the center core, but this cannot be validated by the lack of data from the north core. Despite the relative low impact in the total community of the wet period identified at ~3000 years BP, a shift in the population of sulfur oxidizers was observed. While the samples NC23, NC15 and NC14 (corresponding to the driest periods) were dominated by *Sulfuricurvum* and *Thiovirga*, samples NC21 and NC22 (wetter period) were dominated by *Thiobacillus*. The large dominance of *Sulfuricurvum* in the center core highlights the difference in the hydrological regime in both core locations, with lower lake level in the center area, as shown by the geochemical data as well. This supports the association of *Sulfuricurvum* to dry (and oxygen-rich) environments, and *Thiobacillus* to wet (oxygen-poor) environments.

2000 to 1000 years BP (wet period)

A humid period beginning at ~2000 years BP was reflected by an increase in smectite abundance, with a very significant smectite peak in 1500 years BP. While in the north core, this humid period is reflected by an increasing lake level, the center core showed a delay in its response with the samples CC10 and CC08 being still associated to the emersion phase. In the north core, accumulation of organic matter with a higher input of terrestrial material and better preservation is consistent with an increase in the terrestrial vegetation productivity due to a more humid climate and decrease in oxygen levels due to an increasing water level. In the center, the progressive reflooding after the emersion phase appeared to attenuate/delay the signal of this humid period, notably in the quality (oxidation) of the organic matter.

During this period, bacteria from the sulfur cycle remain important, but in contrast to the previous period, sulfate-reducing organisms were slightly more abundant in the north and center cores. The dominance of *Thiobacillus* in the north core and *Sulfuricurvum* in the center core tends to confirm that more humid conditions prevail in former as indicated above. Thermophiles also remained abundant in this section, with abundances comparable to what was observed in the preceding period. In addition, sulfate reducers drastically increased in the lysis-resistant fraction (more marked in center than in north core). This was characterized by increasing abundance of *Desulfurisporosinus*, *Desulfurispora* and *Desulfotomaculum* in the center core, and mainly *Desulfurispora* in the north core. The last two genera include thermophilic species (Kaksonen et al., 2007; Schleifer, 2009). Interestingly, this increase corresponds to the period when hydrothermal activity is supposed to be intensive (peak of barium in center core; Figure 12), suggesting a link between hydrothermalism and the sulfur cycling. Detection of thermophilic sulfate-reducing bacteria have already been reported in hot sediment from a hydrothermal vent in a Lake Tanganyika, where the availability of sulfate was attributed to the hydrothermal fluids (Elsgaard et al., 1994).

Surprisingly, no evidence for the development of a soil (or at least the emersion of the sediment) in sample CC10 was reflected in the BCC, while we could expect a major change in the BCC due to the

drastic change in the environmental conditions. Likewise, the transition to a more humid period and a return to dry conditions was not reflected, samples CC05 to CC12 being grouped together. This could be due to both the slow flooding of the lake and the colonization of the sediment after flooding. Although a transition to a humid period was recorded in CC08, the system has been flooded gradually and it is likely that the sediments were periodically re-emerged and the lake level remained low even during wet season. In addition, the flooding of the sediment probably induced a change in the community within the sediment, overlaying the signal of the community hosting the sediment during the emersion period. Giving the low sedimentation rate in the center core, this supposed sediment in situ activity may impact layers representing extended period. This could explain that, neither the hydrological variations, nor a main change in the community in ~2000 years BP, were reflected in the BCC.

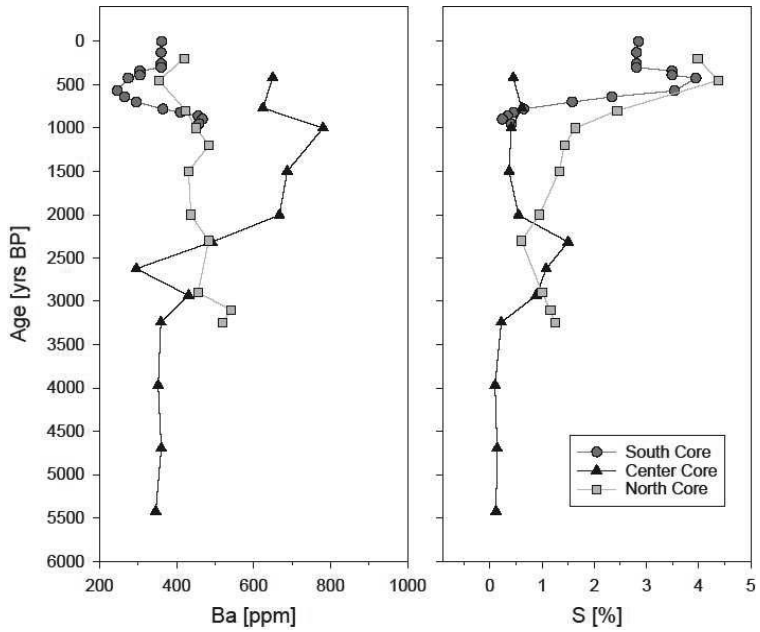

Figure 12: Profiles of barium (Ba) and sulfur (S) along the three cores.

1000 to 600 years BP (dry period)

The overall analysis would suggest that the last millenary could be divided in two periods: a first drier period occurring between 1000 to 600 years BP, and a more recent wet period (or a trend towards more humid conditions) from 600 to present. These two periods could not be distinguished in the north and center cores, probably due to sampling resolution and sedimentation rate. Therefore, the interpretation of the environmental conditions prevailing during the last millenary were based on the south core. The mix origin in the organic matter and the proposition of sand in the section SC23 to SC17 suggest a high energy aquatic system that might correspond to a marsh environment with running water. In the center core, the lack of a clear wet period in the previous section results not in the return to a dry period, but rather into a dry but more humid period (accumulation of organic matter with a mix origin) as compared to the emerged period.

The predominance of sulfur-oxidizing bacteria indicated an active sulfur cycle. As observed in the previous sections, sulfate-reducing bacteria were detected at low abundance, suggesting that sulfide originates from the degradation of organic matter and/or hydrothermal activity. The presence of

Sulfuricurvum testimonies of a non-entirely lacustrine environment, which is in accordance with the geochemical analyses. However, the most relevant sulfur oxidizer is *Thiobacillus*, which elsewhere is linked to wetter conditions, suggesting that despite the dry period indicated by the smectite profile, this period is less dry that the two dry periods described previously.

600 years BP to present (wet period)

The shift to lacustrine conditions (SC10-SC01) is attested by a high level of Corg with a lacustrine origin and low oxidation. In the north core this shift is principally registered in the lacustrine origin (NC02). A decrease in both sulfur-oxidizing and thermophilic bacteria, together with the emergence of unclassified Anaerolinaceae are among the most significant features observed from 600 years BP to the present. All of these changes were observed in the total and lysis-resistant fractions of the bacterial community. The abundance of Anaerolinaceae suggest a decrease in oxygen availability and lacustrine conditions, which is consistent with the description based on geochemical measurements. Sulfur-oxidizing bacteria disappeared almost completely during this period, indicating that the sulfur cycle might be extremely limited. The disappearance of sulfur and iron-oxidizing bacteria in favor of fermentative bacteria suggests the establishment of persistent anoxic conditions. This is confirmed by the increase in pyrite (Supplementary Figure 2). In addition, the decrease of thermophilic bacteria is consistent with a decreasing intensity of hydrothermal events, also suggested by the low amount of barium measured (Figure 12). Interestingly, sulfate-reducing bacteria slightly increased in the upper section of the south core, while they decrease in the lysis-resistant fraction. The hypothesis in this case is that persistent anoxic conditions and the availability of substrates for sulfate reduction might prevail when the water level is high (Holmer 2001). On the contrary, when lake water levels are low, the upper sediment is regularly oxygenated and sulfate reduction is sporadic, leading to sporulation/germination in response to the change in oxygen and substrate availability. In this case, sulfate-reducing bacteria might be better detected in the lysis-resistant fraction.

4.4.8 Speculation about hydrothermal activity in Lake Liambezi

As indicated above, the presence in high relative abundance of numerous thermophilic and moderately thermophilic bacteria, notably in the lowest part of the center core, draws attention to probable hydrothermal activity in the lake. The high abundance of thermophilic bacteria in the total fraction of the BCC suggested hydrothermal activity occurring directly in the lake. The principal component analysis (PCA, Figure 2A-B) showed three groups of minerals and elements linked to a detrital source. However, Barium (Ba) represented a group on its own. This feature was confirmed by a comparison of Ba to the other alkaline earth metals that showed a different origin. The origin of Ba is partially detrital, but a second source is required to explain the content of Ba in the sediments. Local hydrothermal activity is suggested as a secondary source as Ba is typically deposited as a sulfate ($BaSO_4$) during hydrothermal activity.

Based on the combined results of the bacterial community and the Ba levels, a hypothetic history of hydrothermal activity in the lake was proposed. Despite the high abundance of thermophilic bacteria, the low levels of barium suggest low intensity of hydrothermal events during the period ranging from 5500 to 3200 years BP. However, an alternative explanation is that Ba is not precipitated during this period, due to low sulfate concentrations. This is consistent with a low lake level and a fast turnover of organic matter under oxic conditions, resulting in limited sulfur availability, which was attested by the absence of sulfur-oxidizers in this section. During the following period (3200-1000 years BP),

hydrothermal activity is proposed to be important, due to the high abundance of thermophlic bacteria and the increasing level of Ba in the center core. Despite a decrease in the abundance of thermophilic bacteria, they remained abundant (3-4% of the total community). Maximum Ba level was reached in ~1000 years BP, suggesting the highest hydrothermal activity during that period. The high Ba values correspond to low sulfur (Figure 12) and TOC values, reflecting a high turnover of organic matter. This might be the results of oxic conditions due to lower lake level. Sulfur (originated from hydrothermal sources and/or organic matter) might be biologically or chemically oxidized, possibly favoring the precipitation of barite ($BaSO_4$) and gypsum ($CaSO_4$). Afterwards, the hydrothermal activity is supposed to decrease between 1000 and 600 years BP, due to the simultaneous decrease in the abundance of thermophilic bacteria and levels of Ba. Nonetheless, thermophilic bacteria remained abundant in the center core at the same period, and Ba values were clearly higher than for the other two sites. This suggests that despite a probable global decrease in hydrothermal activity, the remaining activity affects preferentially the center site, probably due to its greater proximity of the hydrothermal source as compared to the other two locations. Lacustrine conditions might also decrease the impact of the hydrothermal activity in the north and south basins. The last 600 years saw a slight increase of Ba (values remain low) coinciding with the almost complete disappearance of thermophilic bacteria. Although the decline of thermophiles could suggest a lower intensity of hydrothermal activity, this might reflect an increasing lake level. The impact of hydrothermalism can be expected to be higher in a shallower lake. Interestingly, sulfur-oxidizing bacteria also decrease during the last millenary. The co-absence of sulfur-oxidizing and thermophilic bacteria in the more recent samples supports the contribution of hydrothermal activity as a significant source of sulfur for this system.

4.5 Conclusion

The results of this study demonstrate the applicability of using bacterial DNA (total and/or spores) to identify changes and variability in the environmental conditions. The proposed innovative multidisciplinary approach allowed the reconstruction of the hydrological regime and the climatic variations in Lake Liambezi (Namibia) during the past 5500 years. Changes in the total and lysis-resistant bacterial communities were consistent with other geological proxies and demonstrated their relevance as a paleoecological proxy. First, changes in the bacterial community were related to changes in the hydrological lake regime and reflected the changing environmental conditions (as illustrated by the consistency with the visual characterization and geochemical/sedimentological measurements). Second, both communities (total and lysis-resistant) were not only relevant, but also complementary. Indeed, specific biological processes or extreme events were in some cases only reflected in one fraction of the community. Finally, the analysis of the bacterial community did not only corroborate environmental fluctuation, it also provided relevant information that helped to calibrate the three cores and to define a common timeline and a refined age model, by comparing the community of specific groups of organisms attesting specific elemental cycling (sulfur) or hydrothermal activity.

4.6 References

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4.7 Supplementary material

Supplementary Table 1: 10 most abundant genera in samples from the north core.

North core

Sample	1st genus	2nd genus	3rd genus	4th genus	5th genus
NC02	Anaerolineaceae	Parcubacteria	Betaproteobacteria	Bacteria	Candidate_division_OP3
NC04	Thiobacillus	Anaerolineaceae	Parcubacteria	Betaproteobacteria	Bacteria
NC06	Thiobacillus	Comamonas	Comamonadaceae	Hydrogenophaga	Acidovorax
NC08	Thiobacillus	Comamonas	Comamonadaceae	Acidovorax	Novosphingobium
NC10	Comamonas	Novosphingobium	Pseudarcicella	Comamonadaceae	Thiobacillus
NC11	Comamonas	Comamonadaceae	Acidovorax	Hydrogenophaga	Acidimicrobiales
NC14	Comamonas	Sulfuricurvum	Pseudarcicella	BSV26	Thiovirga
NC15	Sulfuricurvum	Comamonas	Thiovirga	Acidobacteria_2	Candidate_division_OP3
NC20	Acinetobacter	Betaproteobacteria	Comamonadaceae	Acidobacteria_2	Thiobacillus
NC21	Comamonas	Betaproteobacteria	Acidobacteria_2	Comamonadaceae	Chloroflexi
NC23	Betaproteobacteria	Thiovirga	Acidobacteria_2	Candidate_division_OP3	BSV26

Sample	6th genus	7th genus	8th genus	9th genus	10th genus
NC02	Bacteroidetes_vadinHA17	OPB35_soil_group	Burkholderiales	WCHB1-69	BSV26
NC04	Spirochaeta_2	Bacteroidetes_vadinHA17	Chloroflexi	Comamonadaceae	BSV26
NC06	Gaiellales	Spirochaeta_2	DA111	Nitrosomonadaceae	Chloroflexi
NC08	Hydrogenophaga	BSV26	DA111	Gaiellales	Spirochaeta_2
NC10	Hydrogenophaga	Acidovorax	BSV26	Candidatus_Koribacter	DA111
NC11	DA111	Novosphingobium	Thermoanaerobaculum	Thiobacillus	Bacteria
NC14	Parcubacteria	Thiobacillus	Acidobacteria_2	Candidate_division_OP3	Comamonadaceae
NC15	BSV26	Comamonadaceae	Pseudarcicella	Thermoanaerobaculum	Parcubacteria
NC20	Novosphingobium	Comamonas	BSV26	Chloroflexi	Candidate_division_OP3
NC21	Dyella	Alicyclobacillus	JG37-AG-4	Thermoanaerobaculum	Aeromonas
NC23	Halothiobacillus	Parcubacteria	Chloroflexi	Aeromonas	Arthrobacter

Sample	1st genus	2nd genus	3rd genus	4th genus	5th genus
NC02x	Clostridium_1	Peptostreptococcaceae	Anaerolineaceae	Clostridiaceae_1	Bacillus
NC06x	Alicyclobacillus	Bacteria	Pullulanibacillus	Bacillus	Gaiellales
NC08x	Desulfurispora	Pelotomaculum	Alicyclobacillus	Bacteria	Thiobacillus
NC10x	Arthrobacter	Comamonas	Alicyclobacillus	Desulfurispora	Pelotomaculum
NC11x	Comamonas	Alicyclobacillus	Arthrobacter	Acidimicrobiales	Comamonadaceae
NC14x	Arthrobacter	Acidimicrobiales	Alicyclobacillus	Clostridia	Bacteria
NC15x	Arthrobacter	Alicyclobacillus	Clostridia	Bacteria	Chloroflexi
NC20x	Arthrobacter	Clostridia	Bacteria	Alicyclobacillus	D8A-2
NC21x	Arthrobacter	Clostridia	Bacteria	Alicyclobacillus	Gaiellales
NC23x	Arthrobacter	Clostridia	Bacteria	Alicyclobacillus	Firmicutes

Sample	6th genus	7th genus	8th genus	9th genus	10th genus
NC02x	Chloroflexi	Bacteria	Alicyclobacillus	KD4-96	Burkholderiales
NC06x	Pelotomaculum	Comamonadaceae	Thiobacillus	Comamonas	Desulfurispora
NC08x	Comamonadaceae	Bacillus	Gaiellales	Clostridia	Comamonas
NC10x	Bacteria	Novosphingobium	Clostridia	DA111	Acidimicrobiales
NC11x	Clostridia	Bacteria	Pelotomaculum	Novosphingobium	Desulfurispora
NC14x	Gaiellales	Comamonas	Pelotomaculum	Chloroflexi	Thermoanaerobaculum
NC15x	Comamonas	Acidimicrobiales	Firmicutes	Gaiellales	D8A-2
NC20x	TSAC18	Polaromonas	Firmicutes	Sulfuricella	Ruminiclostridium_1
NC21x	Firmicutes	Chloroflexi	Sulfobacillus	JG37-AG-4	Bacillus
NC23x	Pelotomaculum	Chloroflexi	Gaiellales	Ruminiclostridium_1	JG37-AG-4

Supplementary Table 2: 10 most abundant genera in samples from the center core.

Center core

Sample	1st genus	2nd genus	3rd genus	4th genus	5th genus
CC02	Sulfuricurvum	Halothiobacillus	Comamonadaceae	Parcubacteria	Betaproteobacteria
CC03	Sulfuricurvum	Comamonadaceae	Halothiobacillus	Betaproteobacteria	Thiobacillus
CC05	Sulfuricurvum	Comamonadaceae	Gaiellales	Parcubacteria	Hydrogenophaga
CC08	Sulfuricurvum	Betaproteobacteria	Acidovorax	Comamonadaceae	Bacteria
CC10	Sulfuricurvum	Hydrogenophaga	Comamonadaceae	Ignavibacterium	Bacteria
CC12	Sulfuricurvum	Comamonadaceae	Hydrogenophaga	Bacteria	Arthrobacter
CC14	Comamonadaceae	Sulfuricurvum	Aeromonas	Hydrogenophaga	Spirochaeta_2
CC24	Alicyclobacillus	BSV26	Thermobacillus	Bacteria	Ignavibacteriales
CC27	Thermobacillus	Acidobacteria_2	Ignavibacteriales	Bacteria	Acidimicrobiales
CC29	Alcaligenaceae	Bacteria	Acidobacteria_2	Thermobacillus	Ignavibacteriales

Sample	6th genus	7th genus	8th genus	9th genus	10th genus
CC02	Anaerolineaceae	Thiobacillus	Nitrospiraceae	Bacteria	Chloroflexi
CC03	Nitrospiraceae	Anaerolineaceae	Parcubacteria	Bacteria	Aeromonas
CC05	JG37-AG-4	Chloroflexi	Bacteria	Gemmatimonadaceae	Anaerolineaceae
CC08	Hydrogenophaga	Alcaligenaceae	Thermoanaerobaculum	Aeromonas	Chloroflexi
CC10	Acidovorax	Gallionellaceae	Parcubacteria	Chlamydiales	Thermoanaerobaculum
CC12	Candidate_division_OP3	Parcubacteria	Acidovorax	Aeromonas	Spirochaeta_2
CC14	Paenibacillus	Candidate_division_OP3	Bacteroidetes_vadinHA17	BSV26	Vogesella
CC24	Candidate_division_OP3	Bacteroidetes_vadinHA17	Parcubacteria	Dehalococcoidia	Acidobacteria_2
CC27	BSV26	Thermoanaerobaculum	Dehalococcoidia	Candidate_division_OP3	Candidatus_Solibacter
CC29	Candidate_division_OP3	Thermoanaerobaculum	Enterobacteriaceae	Dehalococcoidia	Subgroup_13

Sample	1st genus	2nd genus	3rd genus	4th genus	5th genus
CC02x	Arthrobacter	Bacillus	Alicyclobacillus	Pullulanibacillus	Tumebacillus
CC03x	Arthrobacter	Bacillus	Desulfosporosinus	Oxalophagus	OPB54
CC05x	Clostridium_12	Desulfosporosinus	Ruminiclostridium_1	Desulfurispora	Clostridium_1
CC08x	Desulfosporosinus	Bacteria	OPB54	Alcaligenaceae	Bacillus
CC10x	Bacteria	Bacillus	Desulfosporosinus	Alicyclobacillus	Gallionellaceae
CC12x	Acidimicrobiales	Gaiellales	Comamonadaceae	Hydrogenophaga	JG37-AG-4
CC14x	Clostridia	OPB54	Comamonadaceae	Aeromonas	Alicyclobacillus
CC17x	Acidimicrobiales	Clostridia	Comamonadaceae	Enterobacteriaceae	Aeromonas
CC20x	Clostridia	Acidimicrobiales	Bacteria	Alcaligenaceae	Alicyclobacillus

Sample	6th genus	7th genus	8th genus	9th genus	10th genus
CC02x	Acidothermus	Bacteria	Nocardioides	Sulfobacillus	OPB54
CC03x	Alicyclobacillus	Pullulanibacillus	Acidothermus	Ruminiclostridium_1	Desulfurispora
CC05x	Bacillus	Oxalophagus	Desulfotomaculum	Peptococcaceae	Clostridia
CC08x	Dehalococcoidia	Peptococcaceae	Planctomycetaceae	Chloroflexi	Desulfurispora
CC10x	Peptococcaceae	Clostridia	Mycobacterium	Ruminococcaceae	Desulfitobacterium
CC12x	Thermoanaerobaculum	Aciditerrimonas	Sulfuricurvum	Gemmatimonadaceae	DA111
CC14x	Acidothermus	Sulfobacillus	Bacteria	Acidimicrobiales	Hydrogenophaga
CC17x	Alphaproteobacteria	Bacteria	Paenibacillus	Hydrogenophaga	JG37-AG-4
CC20x	Dehalococcoidia	Gaiellales	Comamonadaceae	Firmicutes	Burkholderia

Supplementary Table 3: 10 most abundant genera in samples from the south core.

South core

Sample	1st genus	2nd genus	3rd genus	4th genus	5th genus
SC01	Anaerolineaceae	WCHB1-69	Bacteria	Parcubacteria	Bacteroidetes_vadinHA17
SC02	Anaerolineaceae	Xanthomonadales	SZB30	Bacteria	Burkholderiales
SC05	Bacillus	Anaerolineaceae	Bacteria	Xanthomonadales	Candidate_division_OP3
SC06	Anaerolineaceae	SZB30	Xanthomonadales	Candidate_division_OP3	Bacillus
SC08	Anaerolineaceae	Chloroflexi	SZB30	Nitrospiraceae	Bacteria
SC10	4-29	Anaerolineaceae	Bacteria	Nitrospiraceae	Bacillus
SC12	Thiobacillus	Bacillus	Anaerolineaceae	4-29	Nitrospiraceae
SC16	Thiobacillus	Anaerolineaceae	Bacillus	Bacteria	Nitrospiraceae
SC17	Thiobacillus	Gallionellaceae	Bacillus	Sulfuricurvum	Gemmatimonadaceae
SC18	Thiobacillus	Bacillus	Anaerolineaceae	Gemmatimonadaceae	Spirochaeta_2
SC19	Thiobacillus	Sulfuricurvum	Gemmatimonadaceae	Comamonadaceae	Spirochaeta_2
SC20	Thiobacillus	Bacillus	Gemmatimonadaceae	Sulfuricurvum	Anaerolineaceae
SC21	Thiobacillus	Gemmatimonadaceae	Comamonadaceae	Nitrosomonadaceae	Gemmatimonas
SC22	Thiobacillus	Sulfuricurvum	Comamonadaceae	Gemmatimonadaceae	Aquabacterium

Sample	6th genus	7th genus	8th genus	9th genus	10th genus
SC01	Candidate_division_OP3	BSV26	Xanthomonadales	Nitrospiraceae	SZB30
SC02	Candidate_division_OP3	Chloroflexi	Parcubacteria	WCHB1-41	Betaproteobacteria
SC05	SZB30	Burkholderiales	Parcubacteria	OPB35_soil_group	Chloroflexi
SC06	Bacteria	Burkholderiales	Chloroflexi	Nitrospiraceae	Parcubacteria
SC08	Burkholderiales	Xanthomonadales	Bacillus	Anaeromyxobacter	Candidatus_Competibacter
SC10	Anaeromyxobacter	Desulfatiglans	Parcubacteria	SZB30	Burkholderiales
SC12	Anaeromyxobacter	Bacteria	SZB30	Burkholderiales	Gallionellaceae
SC16	4-29	SZB30	Candidate_division_OP3	Parcubacteria	Bacteroidetes_vadinHA17
SC17	Spirochaeta_2	Anaerolineaceae	Bacteria	Candidate_division_OP3	Bacteroidetes_vadinHA17
SC18	Gallionellaceae	Nitrosomonadaceae	Comamonadaceae	Aminicenantes	Alcaligenaceae
SC19	Bacillus	Betaproteobacteria	Nitrosomonadaceae	Chloroflexi	Anaerolineaceae
SC20	Nitrosomonadaceae	Ramlibacter	Spirochaeta_2	Subgroup_6	Comamonadaceae
SC21	Spirochaeta_2	Pseudolabrys	Candidatus_Koribacter	Ramlibacter	Alicyclobacillus
SC22	Acidovorax	Comamonas	Candidatus_Koribacter	Betaproteobacteria	Novosphingobium

Sample	1st genus	2nd genus	3rd genus	4th genus	5th genus
SC01x	Chloroflexi	Anaerolineaceae	Peptostreptococcaceae	KD4-96	Clostridium_1
SC02x	Anaerolineaceae	Clostridium_1	Chloroflexi	KD4-96	Peptostreptococcaceae
SC05x	Peptostreptococcaceae	Clostridium_1	Clostridiaceae_1	Bacillus	Chloroflexi
SC12x	Peptostreptococcaceae	Clostridium_1	Anaerolineaceae	Clostridiaceae_1	Coriobacteriaceae
SC16x	Anaerolineaceae	Coriobacteriaceae	Chloroflexi	Clostridium_1	KD4-96
SC17x	Bacillus	Fictibacillus	Jeotgalibacillus	Clostridium_1	Peptostreptococcaceae
SC18x	Bacillus	Fictibacillus	Thiobacillus	Alcaligenaceae	Anaerolineaceae
SC19x	Gemmatimonadaceae	Thiobacillus	JG30-KF-AS9	Bacillus	Gaiellales
SC20x	Bacillus	Thiobacillus	Fictibacillus	Tumebacillus	Clostridium_1
SC21x	Thiobacillus	Gemmatimonadaceae	Bacillus	Gaiellales	Alicyclobacillus
SC22x	Thiobacillus	Alicyclobacillus	Bacillus	Gaiellales	Comamonadaceae
SC23x	Alicyclobacillus	Fonticella	Pelotomaculum	Bacillus	Sulfuricurvum

Sample	6th genus	7th genus	8th genus	9th genus	10th genus
SC01x	Coriobacteriaceae	Gaiellales	Planctomycetaceae	SZB30	MB-A2-108
SC02x	Coriobacteriaceae	Gaiellales	SZB30	Xanthomonadales	Planctomycetaceae
SC05x	Anaerolineaceae	KD4-96	Coriobacteriaceae	Gaiellales	Planctomycetaceae
SC12x	Chloroflexi	Bacillus	OPB54	Bacteria	KD4-96
SC16x	Peptostreptococcaceae	Thiobacillus	Anaeromyxobacter	Gaiellales	Bacillus
SC17x	Paenibacillus	Gemmatimonadaceae	Clostridiaceae_1	Alicyclobacillus	Anaerolineaceae
SC18x	Clostridium_1	Gemmatimonadaceae	Subgroup_6	Alicyclobacillus	SC-I-84
SC19x	Nitrosomonadaceae	Gemmatimonas	Alicyclobacillus	Fictibacillus	Comamonadaceae
SC20x	Alicyclobacillus	Gemmatimonadaceae	Paenibacillus	Bacteria	Oxalophagus
SC21x	Nitrosomonadaceae	Gemmatimonas	Comamonadaceae	JG30-KF-AS9	Chloroflexi
SC22x	Sulfuricurvum	Gemmatimonadaceae	Nitrosomonadaceae	Fictibacillus	Bacteria
SC23x	OPB54	Sulfobacillus	Ruminiclostridium_1	Oxalophagus	Bacteria

Supplementary Table 4: SIMPER analysis on samples group from the north core.

NORTH CORE: TOTAL

NC3_total_NC2_total

	OTU	Genus	average	sd	ratio	ava	avb	cumsum
1	Otu000008	Comamonas	0.01017769	0.0023292	4.36961547	0.03770422	0.3118142	0.0174481
2	Otu000014	Novosphingobium	0.00672925	0.00275626	2.4414374	0.02327155	0.20379912	0.02898436
3	Otu000005	Comamonadaceae_unclass.	0.00654429	0.00318928	2.05196191	0.08908104	0.26346202	0.04020354
4	Otu000017	Acidovorax	0.00576865	0.00249625	2.3109296	0.05085247	0.20489782	0.05009301
5	Otu000013	Hydrogenophaga	0.00559252	0.00238906	2.34089076	0.04950336	0.19847933	0.05968053
6	Otu000855	Parcubacteria_unclass.	0.00497484	0.00187312	2.65591194	0.15894013	0.02608098	0.06820913
7	Otu000042	Burkholderiales_unclass.	0.00487346	0.00114462	4.25770622	0.17398366	0.0426605	0.07656393
8	Otu000045	Pseudarcicella	0.00483554	0.00379309	1.27482826	0	0.1295054	0.08485373
9	Otu000033	Xanthomonadales_unclass.	0.00472149	0.00132377	3.56669645	0.18737362	0.06034712	0.09294801
10	Otu000003	Thiobacillus	0.00454589	0.00391922	1.15989718	0.22739355	0.30730672	0.10074124

NC3_total_NC1_total

	OTU	Genus	average	sd	ratio	ava	avb	cumsum
1	Otu000008	Comamonas	0.0114653	0.00595838	1.92423254	0.03770422	0.31849027	0.01532137
2	Otu000033	Xanthomonadales_unclass.	0.00744215	0.00118447	6.28311701	0.18737362	0.00384919	0.0252665
3	Otu000064	Betaproteobacteria_unclass.	0.00740045	0.00407331	1.81681481	0.00822774	0.19045925	0.0351559
4	Otu000018	Acidobacteria_unclass.	0.00728389	0.00269478	2.70296365	0.03947611	0.21860533	0.04488954
5	Otu000042	Burkholderiales_unclass.	0.0068703	0.00110393	6.2235158	0.17398366	0.00455684	0.05407049
6	Otu000040	Thiovirga	0.00633704	0.00357671	1.77175245	0.02176854	0.17198046	0.06253882
7	Otu000855	Parcubacteria_unclass.	0.00610756	0.00203206	3.00560027	0.15894013	0.01008426	0.0707005
8	Otu000025	BSV26_unclass.	0.00568137	0.00161238	3.52359246	0.01686184	0.15728092	0.07829265
9	Otu000172	Betaproteobacteria_unclass.	0.00567297	0.00091959	6.16902985	0.15946408	0.01945046	0.08587358
10	Otu000807	Betaproteobacteria_unclass.	0.00559314	0.00131626	4.24926059	0.14241917	0.00522069	0.09334784

NC2_total_NC1_total

	OTU	Genus	average	sd	ratio	ava	avb	cumsum
1	Otu000003	Thiobacillus	0.00655576	0.00308538	2.12478536	0.30730672	0.13191549	0.0179048
2	Otu000018	Acidobacteria_unclass.	0.00508228	0.0022461	2.26271541	0.08369342	0.21860533	0.03178527
3	Otu000009	Sulfuricurvum	0.004536	0.00387171	1.17157457	0.07909148	0.14970446	0.04417378
4	Otu000064	Betaproteobacteria_unclass.	0.00452614	0.0036338	1.24556471	0.07352107	0.19045925	0.05653536
5	Otu000045	Pseudarcicella	0.00437389	0.00333921	1.3098558	0.1295054	0.07951579	0.06848111
6	Otu000017	Acidovorax	0.00433905	0.00228295	1.90063407	0.20489782	0.09448805	0.08033171
7	Otu000013	Hydrogenophaga	0.00395538	0.00204601	1.93321514	0.19847933	0.09652158	0.09113445
8	Otu000008	Comamonas	0.00372204	0.00314817	1.18228488	0.3118142	0.31849027	0.10129991
9	Otu000040	Thiovirga	0.00363285	0.00212643	1.70842756	0.11232795	0.17198046	0.11122177
10	Otu000086	Chloroflexi_unclass.	0.00352338	0.00243881	1.44471159	0.03818301	0.12966587	0.12084466

NORTH CORE: LYSIS-RESISTANT

NC3_spores_NC2_spores

	OTU	Genus	average	sd	ratio	ava	avb	cumsum
1	Otu000010	Clostridium_sensu_stricto_1	0.00955302	0.0015099	6.32692565	0.38111861	0.07062473	0.01690833
2	Otu000006	Peptostreptococcaceae_unclass.	0.00807286	0.00195099	4.13782231	0.34237469	0.08069795	0.03119686
3	Otu000008	Comamonas	0.0069411	0.00332528	2.08737567	0	0.22378935	0.04348223
4	Otu000021	Clostridiaceae_1_unclass.	0.00605006	0.00094102	6.42924619	0.25032241	0.05367385	0.05419051
5	Otu000001	Arthrobacter	0.0060031	0.00620546	0.96739037	0.03476773	0.22535231	0.06481568
6	Otu000005	Comamonadaceae_unclass.	0.00545325	0.00087609	6.22450825	0	0.17816941	0.07446764
7	Otu000013	Hydrogenophaga	0.00424289	0.00081834	5.18476146	0.00571578	0.14343583	0.08197733
8	Otu000017	Acidovorax	0.00385487	0.00100242	3.84557952	0.00808333	0.13299062	0.08880023
9	Otu000014	Novosphingobium	0.0035755	0.00203264	1.7590405	0.00808333	0.12269364	0.09512867
10	Otu000138	Turicibacter	0.00354996	0.00047917	7.40862097	0.13753547	0.02207555	0.1014119

NC3_spores_NC1_spores

	OTU	Genus	average	sd	ratio	ava	avb	cumsum
1	Otu000001	Arthrobacter	0.01650903	0.0047058	3.50822902	0.03476773	0.53868257	0.02467941
2	Otu000010	Clostridium_sensu_stricto_1	0.01123566	0.00122619	9.16307911	0.38111861	0.03536514	0.04147563
3	Otu000006	Peptostreptococcaceae_unclass.	0.00991174	0.00127455	7.77667513	0.34237469	0.03760888	0.05629272
4	Otu000021	Clostridiaceae_1_unclass.	0.00728773	0.00099698	7.30979005	0.25032241	0.02629736	0.06718717
5	Otu000033	Xanthomonadales_unclass.	0.00426393	0.00040319	10.5755688	0.1363426	0.00503018	0.07356134
6	Otu000042	Burkholderiales_unclass.	0.0042331	0.00031699	13.3539797	0.1340469	0.00350466	0.07988941
7	Otu000138	Turicibacter	0.00407539	0.00048081	8.47612862	0.13753547	0.01215907	0.08598173
8	Otu000056	SZB30_unclass.	0.00375771	0.00031077	12.0916894	0.11755622	0.00176004	0.09159915
9	Otu000002	Alcaligenaceae_unclass.	0.0037198	0.00067168	5.53804154	0.1351392	0.01979407	0.0971599
10	Otu000065	OM1_clade_unclass.	0.00367577	0.00031199	11.7815272	0.13331373	0.01993436	0.10265481

NC2_spores_NC1_spores

	OTU	Genus	average	sd	ratio	ava	avb	cumsum
1	Otu000001	Arthrobacter	0.00895066	0.00517549	1.72943262	0.22535231	0.53868257	0.02310561
2	Otu000008	Comamonas	0.00374774	0.00288765	1.29785333	0.22378935	0.09007017	0.0327802
3	Otu000005	Comamonadaceae_unclass.	0.00337513	0.00086056	3.92203872	0.17816941	0.05544445	0.04149291
4	Otu000013	Hydrogenophaga	0.0031786	0.00071772	4.42875018	0.14343583	0.02872227	0.04969829
5	Otu000003	Thiobacillus	0.00296865	0.00167351	1.77390919	0.14648009	0.03822863	0.05736168
6	Otu000017	Acidovorax	0.00286534	0.00122331	2.3422852	0.13299062	0.02953504	0.06475839
7	Otu000034	DA111_unclass.	0.00250347	0.00058695	4.26524104	0.12804647	0.0374862	0.07122096
8	Otu000014	Novosphingobium	0.00211282	0.0016224	1.3022823	0.12269364	0.04859575	0.07667509
9	Otu000011	Acidimicrobiales_unclass.	0.00201052	0.00171076	1.17521798	0.11194293	0.12156015	0.08186513
10	Otu000057	Desulfurispora	0.0019659	0.00098085	2.00427192	0.12446245	0.05339881	0.08693998

Supplementary Table 5: SIMPER analysis on samples group from the center core.

CENTER CORE: TOTAL

CC3_total_CC2_total

	OTU	Genus	average	sd	ratio	ava	avb	cumsum
1	Otu000161	Halothiobacillus	0.01230295	0.00095727	12.8521367	0.28712188	0.00972893	0.02720231
2	Otu000013	Hydrogenophaga	0.00597291	0.00127858	4.67153129	0.08987091	0.2246378	0.04040865
3	Otu000017	Acidovorax	0.00539874	0.00278339	1.93962817	0.04332404	0.16533157	0.05234549
4	Otu000437	Betaproteobacteria_unclass.	0.00533373	0.00093423	5.70919784	0.11329822	0.05611737	0.06413858
5	Otu000963	Thiofaba	0.00530066	0.00060484	8.76373917	0.1276599	0.00825895	0.07585855
6	Otu000009	Sulfuricurvum	0.00517736	0.00233499	2.21729337	0.54440545	0.42766802	0.08730589
7	Otu000003	Thiobacillus	0.00507325	0.00153358	3.30810019	0.19546798	0.08107675	0.09852304
8	Otu000038	Alicyclobacillus	0.00461285	0.00145	3.18127821	0.14370602	0.03955565	0.10872224
9	Otu000263	4-29_unclass.	0.0045269	0.00036082	12.5461521	0.10683114	0.00477489	0.11873139
10	Otu000156	Ignavibacterium	0.00410699	0.00304416	1.34913728	0.02067161	0.11390713	0.12781211

CC3_total_CC1_total

	OTU	Genus	average	sd	ratio	ava	avb	cumsum
1	Otu000009	Sulfuricurvum	0.02383684	0.00053499	44.555302	0.54440545	0.01731468	0.03359684
2	Otu000031	Thermobacillus	0.01280871	0.00346453	3.69709496	0.03523321	0.31735868	0.05165009
3	Otu000161	Halothiobacillus	0.01275752	0.00069224	18.4292382	0.28712188	0.00517158	0.06963117
4	Otu000018	Acidobacteria_unclass.	0.01157884	0.00319577	3.62317897	0.01924648	0.27427285	0.08595096
5	Otu000048	Ignavibacteriales_unclass.	0.01116323	0.00121567	9.18276535	0.01389116	0.26042991	0.10168499
6	Otu000005	Comamonadaceae_unclass.	0.01021774	0.00137143	7.45041767	0.24878075	0.02322523	0.11608639
7	Otu000103	Bacteria_unclass.	0.00888135	0.00047445	18.7194548	0.01208996	0.20837331	0.12860421
8	Otu000003	Thiobacillus	0.00861809	0.00029653	29.0628818	0.19546798	0.00489557	0.14075099
9	Otu000020	Thermoanaerobaculum	0.006844	0.00228018	3.00150964	0.03844051	0.18951769	0.15039726
10	Otu000019	Aeromonas	0.0062524	0.00075087	8.32686777	0.14817221	0.01011221	0.15920971

CC2_total_CC1_total

	OTU	Genus	average	sd	ratio	ava	avb	cumsum
1	Otu000009	Sulfuricurvum	0.01756787	0.00226231	7.76546318	0.42766802	0.01731468	0.03289562
2	Otu000031	Thermobacillus	0.0121924	0.00313232	3.89245074	0.03354625	0.31735868	0.05572575
3	Otu000005	Comamonadaceae_unclass.	0.01112416	0.00283382	3.92549766	0.28274319	0.02322523	0.0765556
4	Otu000013	Hydrogenophaga	0.00953967	0.00120028	7.9478928	0.2246378	0.00162385	0.09441853
5	Otu000048	Ignavibacteriales_unclass.	0.00842204	0.00118768	7.09116115	0.06392843	0.26042991	0.1101887
6	Otu000018	Acidobacteria_unclass.	0.00782117	0.0031439	2.48773025	0.09288629	0.27427285	0.12483376
7	Otu000019	Aeromonas	0.00725068	0.0039362	1.84205248	0.17932571	0.01011221	0.13841057
8	Otu000017	Acidovorax	0.00706695	0.00262085	2.69643489	0.16533157	0	0.15164334
9	Otu000038	Alicyclobacillus	0.00669345	0.00465216	1.43878481	0.03955565	0.1972228	0.16417676
10	Otu000103	Bacteria_unclass.	0.00668506	0.00050563	13.2211226	0.05235193	0.20837331	0.17669445

CENTER CORE: LYSIS-RESISTANT

CC3_spores_CC2_spores

	OTU	Genus	average	sd	ratio	ava	avb	cumsum
1	Otu000001	Arthrobacter	0.01743661	0.00422744	4.12462184	0.51165614	0.02486521	0.03305881
2	Otu000004	Bacillus	0.00833055	0.00267279	3.11680088	0.3839978	0.14953057	0.04885305
3	Otu000070	Bacillus	0.00656372	0.00339163	1.93526906	0.23915624	0.05788235	0.06129748
4	Otu000084	Desulfosporosinus	0.00573768	0.00464853	1.23430055	0.06151735	0.21642044	0.0721758
5	Otu000024	Pullulanibacillus	0.00530294	0.00140979	3.7614991	0.19163801	0.0377516	0.08222986
6	Otu000044	Acidothermus	0.00500215	0.00113276	4.415883	0.15718324	0.01336687	0.09171364
7	Otu000060	Clostridium_sensu_stricto_12	0.00479401	0.0059286	0.80862399	0.08526437	0.20616905	0.10080281
8	Otu000127	Ruminiclostridium_1	0.00443707	0.00605565	0.73271629	0.02846636	0.13875488	0.10921524
9	Otu000002	Alcaligenaceae_unclass.	0.00371108	0.00267649	1.38654706	0	0.10922399	0.11625124
10	Otu000076	Alicyclobacillus	0.00344063	0.00135116	2.54641945	0.02404195	0.12334554	0.12277448

CC3_spores_CC1_spores

	OTU	Genus	average	sd	ratio	ava	avb	cumsum
1	Otu000001	Arthrobacter	0.01614803	0.00309807	5.212285	0.51165614	0.02622936	0.02355825
2	Otu000004	Bacillus	0.01126297	0.00216285	5.20747029	0.3839978	0.04575722	0.03998973
3	Otu000070	Bacillus	0.00788071	0.00313683	2.51231717	0.23915624	0.00544389	0.05148684
4	Otu000005	Comamonadaceae_unclass.	0.00770662	0.00225023	3.42481363	0.0163776	0.24813363	0.06272997
5	Otu000011	Acidimicrobiales_unclass.	0.00634343	0.00217164	2.92102798	0.02875281	0.21895918	0.07198436
6	Otu000024	Pullulanibacillus	0.00607327	0.00117972	5.14804854	0.19163801	0.00504677	0.08084462
7	Otu000013	Hydrogenophaga	0.00501145	0.00202706	2.47227463	0.00574624	0.15662858	0.08815578
8	Otu000019	Aeromonas	0.00445238	0.00189387	2.35093465	0.00358468	0.13794122	0.09465132
9	Otu000032	JG37-AG-4_unclass.	0.00407419	0.00088259	4.61616512	0.01001727	0.13290862	0.10059513
10	Otu000062	Alphaproteobacteria_unclass.	0.00386228	0.00169083	2.28424561	0.00206962	0.11873639	0.10622979

CC2_spores_CC1_spores

	OTU	Genus	average	sd	ratio	ava	avb	cumsum
1	Otu000005	Comamonadaceae_unclass.	0.0067655	0.00207177	3.26556198	0.01914137	0.24813363	0.01162034
2	Otu000011	Acidimicrobiales_unclass.	0.00600356	0.00190182	3.15674075	0.01556405	0.21895918	0.02193199
3	Otu000060	Clostridium_sensu_stricto_12	0.00569799	0.00472269	1.20651272	0.20616905	0.02391587	0.03171879
4	Otu000084	Desulfosporosinus	0.00557068	0.00350659	1.58863311	0.21642044	0.03507146	0.04128693
5	Otu000013	Hydrogenophaga	0.004278	0.00186141	2.29825601	0.01210889	0.15662858	0.04863478
6	Otu000127	Ruminiclostridium_1	0.00409463	0.00468062	0.87480387	0.13875488	0.01136146	0.05566767
7	Otu000019	Aeromonas	0.00383276	0.00169852	2.2565317	0.00782901	0.13794122	0.06225078
8	Otu000062	Alphaproteobacteria_unclass.	0.00344803	0.00148376	2.32384343	0.00125424	0.11873639	0.06817307
9	Otu000032	JG37-AG-4_unclass.	0.00339852	0.00089532	3.79587628	0.01787724	0.13290862	0.07401033
10	Otu000004	Bacillus	0.00309828	0.00173684	1.78385688	0.14953057	0.04575722	0.0793319

Supplementary Table 6: Simper analysis on samples group from the south core.

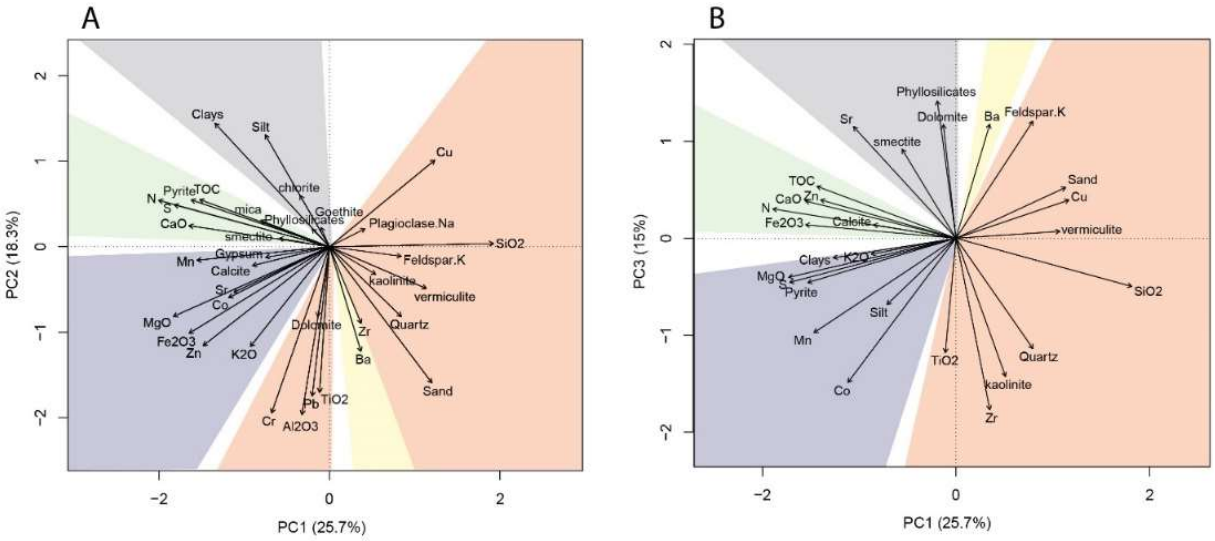
SOUTH CORE: TOTAL

	OTU	Genus	average	sd	ratio	ava	avb	cumsum
1	Otu000003	Thiobacillus	0.01133174	0.00440156	2.57448505	0.13437051	0.49371075	0.01845893
2	Otu000009	Sulfuricurvum	0.00617514	0.00346375	1.7827896	0.02803193	0.22330805	0.02851796
3	Otu000005	Comamonadaceae_unclass.	0.00511418	0.00240356	2.1277546	0.02416438	0.18445414	0.03684875
4	Otu000033	Xanthomonadales_unclass.	0.00434527	0.00109277	3.97639729	0.18354473	0.04489064	0.04392702
5	Otu000056	SZB30_unclass.	0.00396608	0.00083154	4.76956558	0.16181067	0.03537509	0.05038759
6	Otu000042	Burkholderiales_unclass.	0.00395823	0.00073171	5.40959923	0.16550275	0.03930897	0.05683538
7	Otu000004	Bacillus	0.0034723	0.00201659	1.72186368	0.19060449	0.18483813	0.06249161
8	Otu000071	Gallionellaceae_unclass.	0.00346064	0.0026966	1.28333247	0.04916169	0.13119015	0.06812883
9	Otu000147	4-29_unclass.	0.00324524	0.00175954	1.84436619	0.14201412	0.03968157	0.07341519
10	Otu000154	Gemmatimonadaceae_unclass.	0.00315403	0.0011856	2.66027522	0.00142785	0.10230712	0.07855297

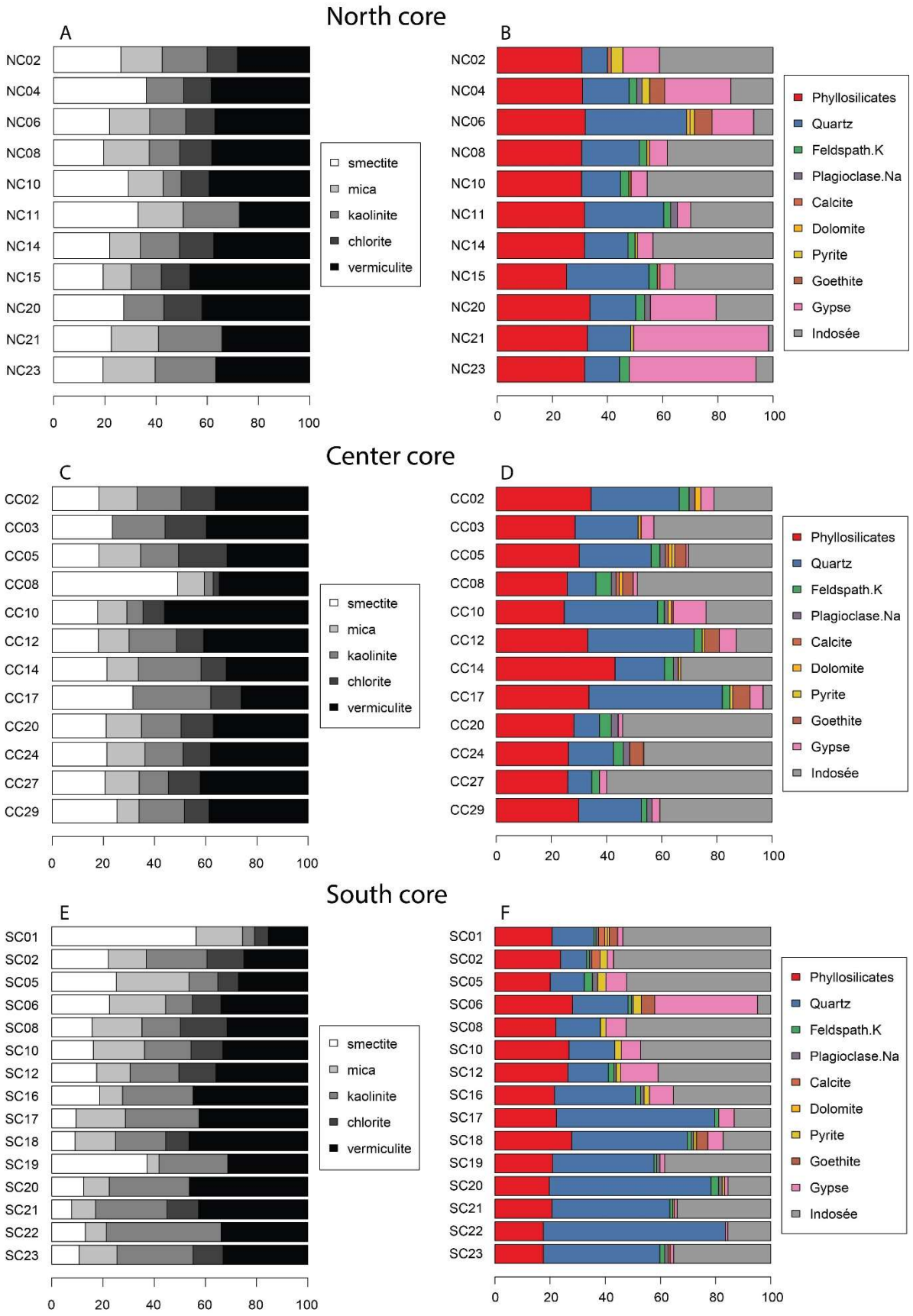
SOUTH CORE: LYSIS-RESISTANT

SC2_spores_SC1_spores

	OTU	Genus	average	sd	ratio	ava	avb	cumsum
1	Otu000006	Peptostreptococcaceae_unclass.	0.00562878	0.00335668	1.6768877	0.31390308	0.09836929	0.01032574
2	Otu000003	Thiobacillus	0.00533402	0.0035914	1.48521872	0.07200849	0.27101633	0.02011076
3	Otu000010	Clostridium_sensu_stricto_1	0.0050602	0.00233352	2.16848507	0.28376629	0.08914716	0.02939348
4	Otu000007	Fictibacillus	0.00453038	0.0035181	1.28773662	0.05087309	0.22719478	0.03770427
5	Otu000039	Coriobacteriaceae_unclass.	0.0033443	0.00098197	3.40570762	0.16845015	0.03792101	0.04383924
6	Otu000021	Clostridiaceae_1_unclass.	0.00256312	0.00153042	1.67478194	0.17174618	0.07323466	0.04854117
7	Otu000004	Bacillus	0.00244101	0.00170665	1.43029803	0.11151568	0.20183356	0.0530191
8	Otu000005	Comamonadaceae_unclass.	0.00229976	0.00146589	1.56885	0.012922	0.10154497	0.05723791
9	Otu000022	Bacillus	0.00225724	0.00165724	1.36205112	0.04247756	0.12760459	0.06137873
10	Otu000110	KD4-96_unclass.	0.00219991	0.0010164	2.16440033	0.11608052	0.0304004	0.06541436



Supplementary Figure 1: Principal component analysis (PCA) illustrating the origin of elements, minerals and organic matter. (A) Axes 1 and 2, (B) axes 1 and 3 (scaling 2).



Supplementary Figure 2: Clay mineralogy (A-C-E) and other major minerals (B-D-F).

5 A historical legacy of antibiotic utilization on bacterial seed banks in sediments

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2018, PeerJ (Vol. 6, e4197), DOI: 10.7717/peerj.4197

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Foreword

During the past decades, the emergence of bacteria resistant to most antibiotics commonly used for medical purpose raised serious concerns about human health in the future. However, little is known about the impact of the extensive use of antibiotics on the accumulation of ARG in the environment, and the role of the environmental pool of ARG on the dissemination of antibiotic resistance. Due to their prevalence in human microbiome and their high dispersal rate, spores and spore-formers have been recently proposed (suggested) to have a significant role in the dissemination of ARG. However, no study was conducted to investigate their ecological (environmental) significance and the prevalence of ARG in spore-formers.

In this chapter, we propose to investigate the accumulation of two ARG in the environment over the past century, using an innovative approach. Taking advantage of the ability of spores to overcome degradation, we evaluated the possible use of DNA extracted from spores as a marker for tracking ARG in sediments.

This publication was a collective effort led by collaborators from our laboratory and other institutions. My personal contribution included part of the laboratory work, the analysis of the sequencing data and the bacterial community, part of the statistical analyses, the integration and interpretation of the results, part of the writing, and the co-supervision of the external collaborator (Laura Madueño) who proceeded to the DNA extractions and collaborated on the quantification of ARG.

A historical legacy of antibiotic utilization on bacterial seed banks in sediments

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ABSTRACT

The introduction of antibiotics for both medical and non-medical purposes has had a positive effect on human welfare and agricultural output in the past century. However, there is also an important ecological legacy regarding the use of antibiotics and the consequences of increased levels of these compounds in the environment as a consequence of their use and disposal. This legacy was investigated by quantifying two antibiotic resistance genes (ARG) conferring resistance to tetracycline (*tet*(W)) and sulfonamide (*sul*1) in bacterial seed bank DNA in sediments. The industrial introduction of antibiotics caused an abrupt increase in the total abundance of *tet*(W) and a steady increase in *sul*1. The abrupt change in *tet*(W) corresponded to an increase in relative abundance from ca. 1960 that peaked around 1976. This pattern of accumulation was highly correlated with the abundance of specific members of the seed bank community belonging to the phylum *Firmicutes*. In contrast, the relative abundance of *sul*1 increased after 1976. This correlated with a taxonomically broad spectrum of bacteria, reflecting *sul*1 dissemination through horizontal gene transfer. The accumulation patterns of both ARGs correspond broadly to the temporal scale of medical antibiotic use. Our results show that the bacterial seed bank can be used to look back at the historical usage of antibiotics and resistance prevalence.

Submitted 19 October 2017
Accepted 5 December 2017
Published 3 January 2018

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Academic editor
Konstantinos Kormas

Additional Information and Declarations can be found on page 13

DOI 10.7717/peerj.4197

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OPEN ACCESS

Subjects Ecology, Microbiology, Health Policy, Public Health, Natural Resource Management
Keywords Antibiotic resistance, Endospores, Clostridia, Tetracycline, Sulfonamide, Sediments, Seed bank

INTRODUCTION

The use of antibiotics to treat infectious diseases represents one of the major scientific achievements of the 20th century. Millions of lives have been saved since the introduction of antibiotics into general medical practice for the treatment of a large range of bacterial infections, as well as other medical procedures (*Marti, Variatza & Balcazar, 2014*). After the initial use of antibiotics in medicine, the utilization of antibiotics to increase agricultural productivity has become a common practice (*Carlet et al., 2011*). Although the positive effect of the so-called antibiotic era on human welfare is not disputed, increased awareness of the risks posed by poor antibiotic stewardship counterbalances this success. Nowadays it

How to cite this article Madueño et al. (2018), A historical legacy of antibiotic utilization on bacterial seed banks in sediments. **PeerJ 6:e4197; DOI 10.7717/peerj.4197**

is becoming clear that the disposal of antibiotics in natural ecosystems can have far-reaching consequences (*Baquero, Martinez & Canton, 2008*). Recent studies on antibiotics and the emergence of resistance suggest that the function of antibiotics in nature cannot be explained solely within the paradigm of chemical weapons in which these compounds have been used since their industrialized production (*Aminov, 2009*; *Aminov, 2010*). Instead, antibiotics and determinants of resistance have been proposed to be a fundamental component of the ecology and evolution of microbial ecosystems. Most of the antibiotics used today are chemical derivatives of small bioactive molecules that might perform a multitude of functions (*Taylor, Verner-Jeffreys & Baker-Austin, 2011*). In nature these molecules are thought to be produced at very low concentrations (*Martinez, 2008*), and for example, a study conducted at sub-inhibitory concentrations with erythromycin and rifampicin has shown that this low concentrations of antibiotics can modulate not only growth but also bacterial metabolism (*Goh et al., 2002*). Therefore, antibiotics can be expected to modulate microbial interactions and regulate the dynamics of microbial communities (*Martinez, 2008*).

Although antibiotic resistance could potentially emerge anywhere and at any given time, the emergence of a resistance factor has been generally associated with some fitness cost, and therefore novel resistance genes are expected to be under strong negative selection pressure (*Bengtsson-Palme, Kristiansson & Larsson, 2017*). In this context, the industrialized production, use, and disposal of antibiotics is a relatively recent phenomenon that has presumably exerted a positive selective pressure for pathogens to develop antibiotic resistance either as a consequence of mutation or by horizontally acquiring naturally occurring antibiotic resistance systems (*Blair et al., 2015*; *Taylor, Verner-Jeffreys & Baker-Austin, 2011*). The increasing levels of antibiotic resistance in bacteria isolated from clinical samples is a problem that threatens health care systems worldwide (*Wright, 2010*). Therefore, understanding the effect of antibiotic use on the natural reservoirs of ARGs and analyzing this recent historical event (the antibiotic era) in terms of the levels of circulating antibiotic resistance genes (ARGs) are essential to develop a management strategy to reduce current and future risks.

ARGs were clearly present in microbial communities before the antibiotic era as shown by phylogenetic analysis of genes conferring resistance to different classes of antibiotics (*Aminov & Mackie, 2007*). Evidence from work conducted on ancient DNA in permafrost (*D'Costa et al., 2011*) and an isolated cave (*Bhullar et al., 2012*) also support the existence of resistance without human intervention. Given the presumed role of human activity in the levels of resistance in the environment, one can thus expect an increasing abundance of such genes in the past century. However, direct evidence for this is currently restricted to a limited number of studies. For example, soil archives from two regions in Europe clearly demonstrate a link between the history of antibiotic use and the increase in the abundance of various genes conferring resistance to a large range of antibiotics (*Graham et al., 2016*; *Knapp et al., 2010*). Furthermore, the analysis of soil records also demonstrated the interconnection between the medical and non-medical use of antibiotics, as well as the effect of changes in policy towards a more strict stewardship in the reduction of ARGs from natural pools (*Graham et al., 2016*).

Besides soils, aquatic ecosystems have been identified as a key ecological component driving the emergence, spread, and persistence of antibiotic resistance (*Baquero, Martinez & Canton, 2008*; *Taylor, Verner-Jeffreys & Baker-Austin, 2011*). Water constitutes a circulating path of antibiotic-resistant organisms from human and animal populations to the environment and back into these populations, via the connection between wastewater treatment and drinking water production, respectively (*Baquero, Martinez & Canton, 2008*). Lake sediments are a major concern because they are a main environmental end-point not only for bacteria, but also for ARGs and antimicrobial agents (*Kümmerer, 2009*). The high numbers of cells in sediments make resuspended sediment material a potential source of resistance determinants. At the same time, lake sediments are natural environmental archives. Thus, the study of the sedimentary record might provide insights into the historical legacy of the antibiotic era and the accumulation of ARG in the environment. Attempts to use DNA extracted from sediments to investigate antibiotic resistance in aquatic systems have been made (*Thevenon et al., 2012*), but suffer from uncertainty regarding the preservation of the environmental signal in the sediments. Sediment microbial communities are strongly shaped by the redox gradients experienced during early diagenesis, and it is therefore unclear how much of the originally resistant community, or of their resistance determinants, is preserved in deeper sediment layers, and how this relationship is affected by environmental factors. The use of microbial seed banks preserved in the sedimentary record as a proxy offers a likely solution to these problems.

The seed bank can be broadly defined as a reservoir of dormant cells that can potentially be resuscitated under favorable environmental conditions (*Lennon & Jones, 2011*). One of the defining features of dormant cells is their reduced metabolic activity (*Driks, 2002*), decreasing the uncertainty generated by environmental changes during sediment diagenesis (*Vuillemin et al., 2016*). In addition, dormant cells are more resistant to degradation than their actively growing counterparts (*Abecasis et al., 2013*). We have used the latter property to develop a specific extraction method to enrich DNA from spores as an example of dormant cell forms (*Wunderlin et al., 2016*; *Wunderlin et al., 2014b*). With this approach we have previously shown that one particular group of bacteria capable of dormancy (endospore-forming *Firmicutes*) can be used as paleoecological biomarkers of the impact of lake eutrophication on microbial communities in sediments (*Wunderlin et al., 2014a*). Using the same selective method we investigated if the historical antibiotic usage has affected the levels of ARG found in the natural seed bank bacterial community. The hypothesis in this case is that information regarding the abundance and frequency of ARGs as the consequence of antibiotic use will be reflected in the dormant cells deposited in the sediment, regardless of the presence of the antibiotics themselves or intrinsic selection by the environment. To test this hypothesis, we investigated the levels of two ARGs conferring resistance to two antibiotics that were introduced earlier in the antibiotic era and with diverging histories of use. The gene *tet*(W) is one of the genes conferring resistance to tetracycline, a class of broad-spectrum antibiotics isolated from *Streptomyces* spp. between 1947 and 1950, constituting one of the earliest classes of antibiotics described and used (*Roberts & Schwarz, 2016*). The second ARG studied here, *sul*1, is one of the genes conferring resistance to sulfonamide drugs, which were also among the earliest

antibiotics discovered. However, in contrast to tetracycline, sulfonamide and its derivatives were obtained by systematic screening of chemically synthesized compounds (*Aminov, 2010*; *Davies & Davies, 2010*). The diverging histories of production and use of these two antibiotics, as well as, the differences in the mechanisms generating resistance, will allow to proof the concept of using the seed bank to investigate the legacy of human antibiotics history, as well as to develop a method to investigate the natural history of antibiotics in the environment.

MATERIAL AND METHODS

Site description and sampling

A sediment core was retrieved with a gravity corer (UWITEC, Mondstein, Au) in August 2011 in an inactive canyon (C1) on the eastern side of the Rhone delta in Lake Geneva (Switzerland) (CAN01, coordinates 559901-139859, 79 m depth, 105 cm). This core has previously been dated by creating an age model based on ^{137}Cs (corresponding to the 1963–1964 atmospheric nuclear tests maximum fallout and the 1986 Chernobyl accident) and magnetic susceptibility, which allowed assigning years to the sediment depth (*Wunderlin et al., 2014a*). Additional environmental data was obtained from a second sediment core (CAN02, 559405-140504, 96 m depth, 107 cm) retrieved in parallel to the sediment used for biological analysis. This second core was split in two lengthwise halves for a sedimentological description and chemical analysis. Manganese and iron measurements were performed at the University of Barcelona by X-ray fluorescence using an AVAATECH XRF core scanner (2000 A, 10 kV and 30 kV) every 2 mm. Correlation between the two sediment cores was carried out by visual description, sediment color and texture and by comparing magnetic susceptibility (MS) and density core profiles in order to assign the manganese and iron profiles to the ages investigated with CAN01 (*Wunderlin et al., 2014a*).

DNA extraction

Total community DNA and DNA from the seed bank were obtained using an indirect extraction method. The extraction of cells from sediments was performed as previously described (*Wunderlin et al., 2013*). The cells extracted from 3 g of wet sediment were filtered onto two different 0.2 μm pore-size nitrocellulose filters (Merck Millipore, Darmstadt, Germany). In one of the filters (1.5 grams of sediment) a treatment to separate seed bank from vegetative cells was performed on the biomass collected on nitrocellulose filters, as previously described (*Wunderlin et al., 2016*; *Wunderlin et al., 2014b*). The first step consisted of the lysis of vegetative cells by heat, enzymatic agents (lysozyme) and chemicals (Tris-EDTA, NaOH, SDS). Further DNase digestion was used to destroy any traces of free DNA. DNA was then extracted from the pre-treated (seed bank DNA) and the second non-pre-treated filter (total community DNA) using a modified protocol with the FastDNA® SPIN kit for soil (MP Biomedicals, Santa Ana, CA, USA) (*Wunderlin et al., 2013*), in which the lysing matrix was submitted to two successive bead-beating steps. Supernatants from each bead-beating step were treated separately downstream according to manufacturer's instructions. The two DNA extracts per filter were pooled by precipitation with 0.3 M Na-acetate and ethanol (99%), stored at −20 °C overnight and centrifuged for

1 h at 21. 460× g and 4 °C. Supernatant was removed and the pellet was washed with 1 volume of 70% ethanol and centrifuged for 30 min at 21.460× g and 4 °C. Supernatant was removed and the residual ethanol was allowed to evaporate at room temperature. DNA was re-suspended in 50 µl of PCR-grade water. DNA was quantified using Qubit® dsDNA HS Assay Kit on a Qubit® 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA). DNA yield varied from 1.6 to 16 µg DNA/g for the total community DNA, and 6–23 ng DNA/g sediment for the seed bank DNA.

Quantitative PCR on *tet*(W) and *sul*1 genes

Quantitative Taqman®-PCR on *sul*1 and *tet*(W) genes was performed in 384-well plates using a LightCycler®480 Instrument II (Roche, Basel, Switzerland). For *sul*1, the primers used were qSUL653f and qSUL719r with tpSUL1 probe (*Heuer & Smalla, 2007*). The reaction mix for *sul*1 consisted of 2 µL of DNA template (between 0.08 and 1.39 ng/µL for seed bank DNA and 10 ng/µL for total community DNA), 0.025 µM of each primer, 0.25 µM of TaqMan probe and 1 × TaqMan® Fast Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA). Total reaction volume of 10 µL was reached with PCR-grade water. For *tet*(W), the primers used were tetW-F and tetW-R with tetW-S probe (*Walsh et al., 2011*). The reaction mix for *tet*(W) consisted of 2 µL of DNA template (between 0.08 and 1.39 ng/µL for seed bank DNA and 15 ng/µL for total community DNA), 0.025 µM of each primer, 0.1 µM of TaqMan probe and 1 × TaqMan®Fast Universal PCR Master Mix (Applied Biosystems, USA). Total reaction volume of 10 µL was reached with PCR-grade water. The qPCR program was the same for both genes and started with a hold at 95 °C for 10 min, followed by 45 cycles of denaturation at 95 °C for 15 s and annealing/elongation at 60 °C for 1 min. The qPCR assays were performed in technical triplicates on samples, standards and negative controls. The negative controls consisted of PCR blanks with only the reaction mix and of PCR blanks containing the mix and 2 µL of PCR-grade water. Standard curves were prepared from serial 10-fold dilutions of plasmid DNA containing the respective target gene in a range of 5×10^7 to 50 gene copies. For *sul*1, control plasmids and standard curves were prepared as previously described (*Heuer & Smalla, 2007*). For *tet*(W), standard curves were prepared as previously described (*Walsh et al., 2011*). The effect of inhibitors on amplification was tested for all the samples and for both genes. All samples were spiked with 10^4 copies of plasmid DNA containing the *tet*(W) or the *sul*1 gene and amplified together with the same set of non-spiked samples and control DNA and the results indicated that inhibition was negligible.

Sequencing and data analysis

Purified DNA extracts were sent to Fasteris (Geneva, Switzerland) for 16S rRNA amplicon sequencing using Illumina MiSeq platform (Illumina, San Diego, CA, USA), generating 250 bp paired-end reads. The hypervariable V3–V4 region was targeted using universal primers Bakt_341F (5′-CCTACGGGNGGCWGCAG-3′) and Bakt_805R (5′-GACTACHVGGGTATCTAATCC-3′) (*Herlemann et al., 2011*). Analysis of the dataset was made using Mothur (*Schloss et al., 2009*) following the standard MiSeq SOP (*Kozich et al., 2013*). The SILVA NR v123 reference database (*Quast et al., 2013*) was used for the

alignment of amplicons and the taxonomic assignment of representative OTUs. After quality filtering and removal of chimeras, a total of 2'837'393 amplicons was obtained (625'339 unique sequences). Singletons were removed prior to the clustering into OTUs. The number of singletons in the dataset was 560'158. Clustering of the 2'277'235 remaining sequences (65'181 unique sequences) was made using a threshold of 97% identity. Finally, 11'802 OTUs constitute the dataset. The generated datasets were submitted to NCBI under the Bioproject accession number PRJNA396276.

Statistical and multivariate analyses

Community and statistical analyses were performed using R version 3.4.0 (*R Core Team, 2014*) and the *phyloseq* and *vegan* packages (*McMurdie & Holmes, 2013*; *Oksanen et al., 2017*). Pairwise correlations between OTU relative abundances and ARGs frequency were calculated using Spearman's rank correlation coefficient. The same analysis was performed using the iron/manganese ratio as a proxy to lake eutrophication. Seed bank community was analyzed by principal coordinates analysis (PCoA), based on Bray–Curtis dissimilarity and Hellinger transformation of the OTUs table (community matrix). Environmental parameters and ARGs abundance/frequency were standardized and passively fitted to the ordination. Only significant parameters were displayed ($p < 0.05$).

RESULTS

Quantification of ARGs in seed bank communities from sediment samples

Seed bank DNA was extracted from a sediment core previously validated for paleoecology covering approximately the last hundred years of sediment accumulation in Lake Geneva (*Wunderlin et al., 2014a*). ARG in seed bank DNA was measured by quantifying the number of copies of genes conferring resistance to tetracycline (*tet*(W) gene) and sulfonamide (*sul*1 gene), two commonly reported antibiotics detected in environmental settings (*Davies & Davies, 2010*). ARG quantification was standardized to DNA yield instead of number of 16S rRNA gene copies given the changes in community composition over time (see next section), and the variable number of copies of this molecular marker in different taxonomic groups (*Lee, Bussema & Schmidt, 2009*). The detection of ARGs in the seed bank DNA changed beginning in 1960 (*tet*(W)) and 1970 (*sul*1). However, the accumulation pattern was different for the two ARGs. In the case of *tet*(W), the total abundance of the gene (copies/g of sediment) increased by an order of magnitude since 1965 compared to the values obtained from 1920 to 1960 (Fig. S1). Moreover, the relative abundance of this ARG (gene copies/ng of DNA) in the seed bank DNA increased from 1961 to 1975 (Fig. 1). In the case of *sul*1, a steady increase of this ARG abundance was observed after 1970 (Fig. S1). The relative abundance of *sul*1 in seed bank DNA increased from the same period, followed by a decline and a more recent increase after the year ca. 2000 (Fig. 1). The specific timeframe in which enrichment in ARG counts per ng of DNA was observed concerned mainly the seed bank DNA, as opposed to the total bacterial community. In addition, we could detect ARGs using a lower initial concentration of DNA for the seed bank community (2 ng of DNA) compared to the total community (10–15 ng of DNA).

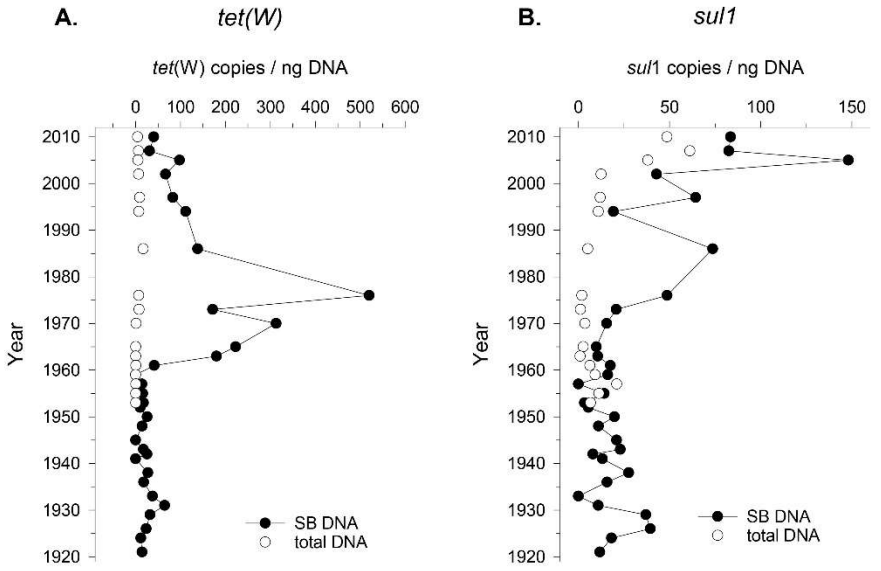

Figure 1 Tetracycline and Sulfonamide resistance in total bacterial community and in the seed bank over time. Relative abundance (gene copies/ng of extracted DNA) of two genes conferring resistance to (A) the antibiotics tetracycline (*tet*(W)) and (B) sulfonamide (*sul*1) in sediment samples covering the period between 1920 and 2010 in Lake Geneva, Switzerland. Quantification was made in DNA extracted from the seed bank (SB DNA) and total microbial community (total DNA).

Full-size DOI: 10.7717/peerj.4197/fig-1

This further suggests a preferential enrichment of ARGs in seed bank bacteria compared to the overall environmental background.

Characterization of the seed bank communities

Previous studies in Lake Geneva have shown a dramatic effect of human activity on the nutritional status of the lake. The lake became eutrophic between 1954 and 1986, and this modified the proportion of some members of the bacterial community in sediments (*Wunderlin et al., 2014a*). Eutrophication is partly related to the same human activities that also shaped the antibiotic era (for example, increased agricultural and livestock output and population pressure). Since changes in microbial community composition as well as the spread of ARG within populations can influence the record of antibiotic resistance, it was important to analyze seed bank community composition alongside ARG quantification. Representatives of six major bacterial phyla (*Proteobacteria, Firmicutes, Actinobacteria, Planctomycetes, Chlamydiae*, and *Chloroflexi*) were the main components of the bacterial seed bank community in sediments (Fig. S2; Fig. 2A). The overall community analysis revealed similarities in the community composition in samples with higher relative abundance of either *tet*(W) or *sul*1 (Fig. 2B). For the former, a significant contribution of OTUs belonging to the Phylum *Firmicutes* was observed, while in the case of *sul*1 no particular bacterial group was correlated with increased accumulation.

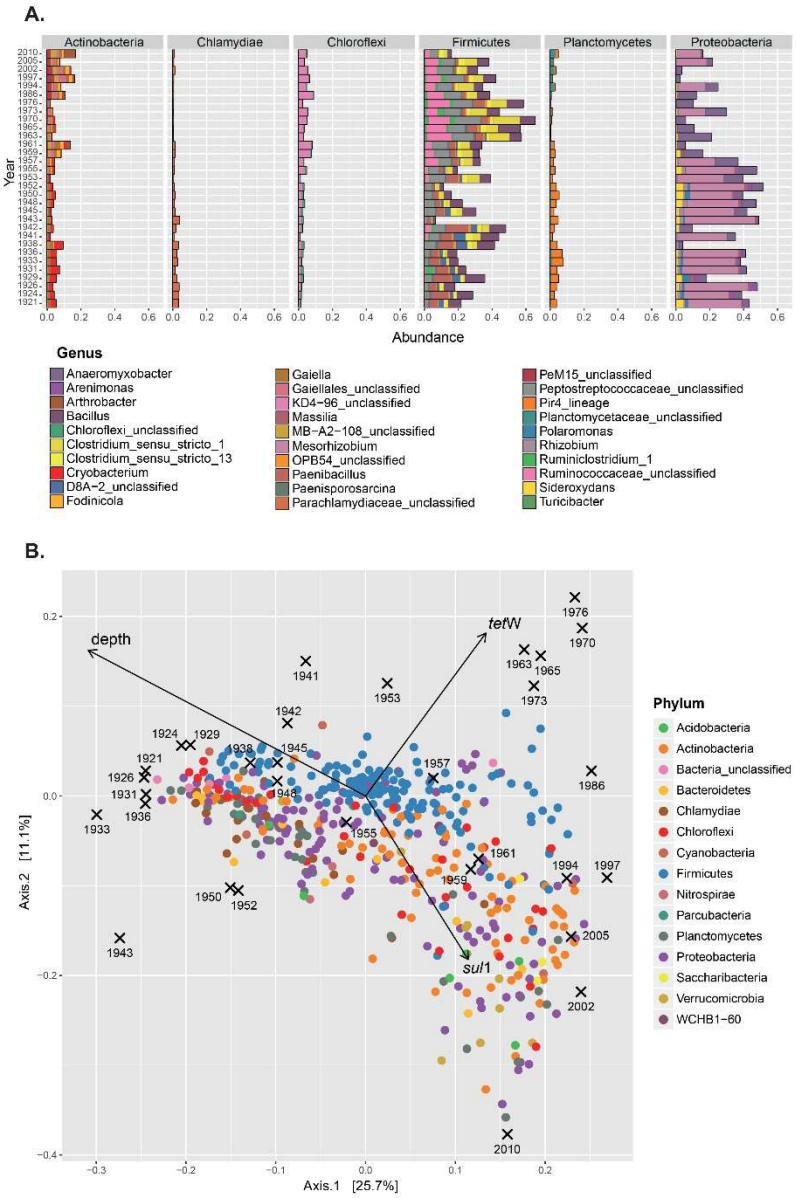

Figure 2 Seed bank community composition in sediments from Lake Geneva. (A) Contribution (relative abundance) of individual genera from the six most abundant bacterial phyla present in the sediment samples. (B) Principal coordinates analysis (PCoA) of the seed bank bacterial community showing the effect of lake eutrophication (Axis 1; depth vector) and the accumulation of ARG (*tet*(W) and *sul*1 vectors).
Full-size DOI: 10.7717/peerj.4197/fig-2

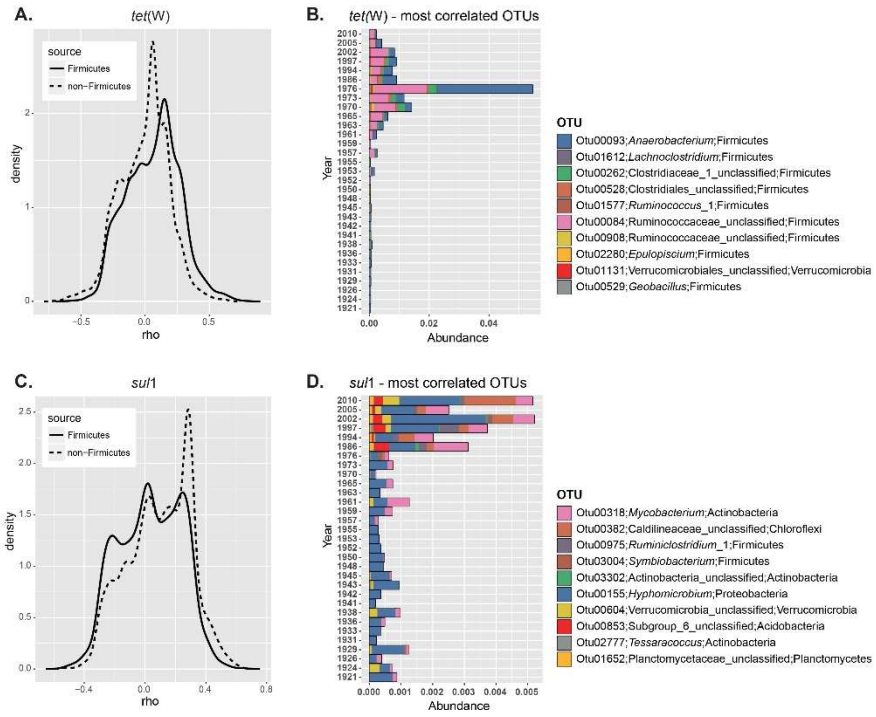

Figure 3 Correlation of specific OTUs to the relative abundance of ARGs in sediments. (A) Spearman correlation coefficients calculated for the relative abundance of each individual OTU and ARG frequency at different depths. The correlation coefficients were plotted as a continuum for the non-Firmicutes seed bank community (dashed line) or the OTUs belonging to Fimicutes only (solid line). (B) Relative abundance of the ten most positively correlated OTUs with the relative abundance of each individual ARG.
Full-size DOI: 10.7717/peerj.4197/fig-3

In order to understand more clearly the relationship between ARG enrichment and seed bank bacterial community, we next studied if the relative abundance of certain OTUs was correlated with ARG levels. For this, we calculated the correlation coefficient between the relative abundance of each OTU and the ARG relative abundance at different depths. Correlation coefficients were plotted as a continuum to analyze the overall response of the community (Fig. 3A). In the case of *tet*(W) most of the non-Firmicutes seed bank community was not correlated with increased ARG relative abundance over time (most correlation coefficients were close to 0; Fig. 3A; dashed line). However, when the analysis is made only for representatives of the Phylum *Firmicutes*, the distribution shifted significantly towards positive correlations (comparison of the distribution for the total and *Firmicutes* communities; $t = 16.52$, $df = 6171.6$, p-value $< 2.2e{-}16$; Fig. 3A; solid line). This analysis confirmed the results of the total community analysis (Fig. 2B). We investigated further the ten most positively correlated OTUs. Nine out of the ten operational taxonomic units (OTUs) positively correlated with *tet*(W) relative

Table 1 Correlation analysis between individual OTUs and relative abundance of *tet* (W) and sul1. Top 10 most positively and negatively correlated OTUs. For *tet* (W) gene, mostly OTUs belonging to *Firmicutes* have been correlated to *tet* (W) abundance. In contrast, for *sul*1, OTUs correlated to *sul*1 abundance belong to many phyla.

Gene	OTU	Phylum	Genus	Correlation coefficient
***tet* (W)**	Otu00093	*Firmicutes*	*Anaerobacterium*	0.7890
	Otu01612	*Firmicutes*	*Lachnoclostridium*	0.7391
	Otu00262	*Firmicutes*	Clostridiaceae 1 unclassified	0.7136
	Otu00528	*Firmicutes*	*Clostridium* unclassified	0.6990
	Otu01577	*Firmicutes*	*Ruminococcus* 1	0.6791
	Otu00084	*Firmicutes*	Ruminococcacea unclassified	0.6722
	Otu00908	*Firmicutes*	Ruminococcacea unclassified	0.6684
	Otu02280	*Firmicutes*	*Epulopiscium*	0.6684
	Out01131	*Verrucomicrobia*	Verrucomicrobiales unclassified	0.6659
	Otu00529	*Firmicutes*	*Geobacillus*	0.6652
***sul*1**	Otu00318	*Actinobacteria*	*Mycobacterium*	0.6656
	Otu00382	*Chloroflexi*	Caldilineaceae unclassified	0.6517
	Otu00975	*Firmicutes*	*Ruminiclostridium* 1	0.6479
	Otu03004	*Firmicutes*	*Symbiobacterium*	0.6341
	Otu03302	*Actinobacteria*	Actinobacteria unclassified	0.6195
	Otu00155	*Proteobacteria*	*Hypomicrobium*	0.6176
	Otu00604	*Verrucomicrobia*	Verrucomicrobia unclassified	0.6170
	Otu00853	*Acidobacteria*	Subgroup 6 unclassified	0.6103
	Otu02777	*Actinobacteria*	*Tessaracoccus*	0.6095
	Otu01652	*Planctoymcetes*	Plactomycetaceae unclassified	0.6092

abundance belong to *Firmicutes* (Table 1). The origin and ecology of bacteria related to those OTUs suggests an equal contribution of bacteria from an environmental origin, mainly cellulose-degrading anaerobic bacteria such as *Anaerobacterium* (*Horino, Fujita & Tonouchi, 2014*) (OTU00093 and OTU00528), *Clostridium* (*Hernandez-Eugenio et al., 2002*; *Miller et al., 2011*; *Zhilina et al., 2005*) (OTU00262, OTU00084, and OTU02280), and *Acetivibrio* (*Patel et al., 1980*) (OTU00908); and from human (or animal) intestinal origin such as *Ruminoccous* (*Cann, Bernardi & Mackie, 2016*; *Chassard et al., 2012*; *Crost et al., 2016*) (OTU01612 and OTU01577). The OTUs positively correlated to *tet* (W) represented a minor fraction of the bacterial seed bank community even for those samples with the highest ARG abundance (relative OTU abundance not higher than 5%; Fig. 3B).

The same analysis performed on *sul*1 showed a larger fraction of the community positively correlated to relative ARG abundance (Fig. 3A), but in contrast to *tet* (W) this is not specifically significant for *Firmicutes* only. Instead, the 10 most positively correlated OTUs belonged to diverse phylogenetic groups (*Actinobacteria, Chloroflexi, Firmicutes, Proteobacteria, Verrucomicrobia*, and *Planctomycetes*) (Table 1). OTUs correlated positively with *sul*1 abundance represented only minor fractions of the seed bank community (Fig. 3A). Interestingly, the correlation coefficients are higher for *tet* (W) than for *sul*1, suggesting a stronger relationship of particular OTUs with the former.

Even though the analysis of the total community suggests that the effect of increased relative abundance of ARG appears to be independent from the generalized effect of eutrophication, we performed the same correlation analysis between relative OTU abundance and the iron/manganese ratio in sediments. The ratio of iron and manganese can be used as a proxy for redox conditions in the water column (*Corella et al., 2012*; *Koinig et al., 2003*) and changes in the relative concentration of these two elements have been shown to correlate with eutrophication in Lake Geneva (*Wunderlin et al., 2014a*). Eutrophication in Lake Geneva is one of the environmental disturbances with the best ecological record. Long-term trends show a steady increase of total phosphorus since 1957 with a peak in 1979. These values, together with phosphate data since 1970, indicate a shift in trophic status of the lake from oligotrophic to eutrophic taking place in the late 1960s. The system has since recovered, even though total phosphorus levels are still double the values before 1960 (*Lazzarotto & Klein, 2012*). The results show no overlap between the overall effect of eutrophication in specific OTUs (Fig. S3) and the effect of ARG abundance in terms of the most correlated OTUs (Fig. 3).

DISCUSSION

Lake Geneva is one of the largest lakes in Europe and constitutes a major reservoir of drinking water. The composition of bacterial communities (*Haller et al., 2011*; *Sauvain et al., 2014*), as well as the presence of toxic metals (*Pote et al., 2008*), micropollutants (*Bonvin et al., 2011*), and ARGs (*Czekalski et al., 2012*; *Czekalski, Gascon Diez & Bürgmann, 2014*; *Devarajan et al., 2015*), has been monitored regularly in its water column and sediments. All these studies have demonstrated the role of human activity in the transfer of contaminants (including antibiotics) into sediments. Because of these preliminary studies, Lake Geneva is an ideal model system to validate the use of the seed bank bacterial community as a proxy to the effect of the historical use of antibiotics on the abundance of ARGs in the environment. Our results show that studying the bacterial seed bank community in sediments of Lake Geneva shows the historical increase in ARG abundance. There was a clear link between seed bank taxonomy and accumulation of *tet*(W). This taxonomy-specific effect has been well documented in the case of tetracycline (*Roberts & Schwarz, 2016*). Tetracycline is a class of broad-spectrum antibiotics active against a wide range of bacteria, including some atypical pathogens such as *Mycoplasma* and *Chlamydia*, and even eukaryotic parasites. In the USA, tetracycline became extensively used in production of livestock between 1950s and 1970s and remains today the second most commonly used antibiotic in agriculture (*Roberts & Schwarz, 2016*). The situation in Switzerland is similar, according to a recent report from the Swiss Federal Office of Public Health indicating that tetracycline (together with penicillin) is the second most sold antibiotic product, after sulfonamides (*FOPH, 2016*). In Switzerland, the current use of tetracycline is mainly restricted to non-medical applications, with a reported consumption below 1% in hospitals (according to data covering the period from 2004 to 2015) and close to 11% in outpatient settings (*FOPH, 2016*). In Switzerland the principal medical use of tetracycline was reported for the period of 1955–1970 (Table S1), but has since reduced dramatically